Triazole-linked Glycosyl Amino Acids and Peptides

Synthesis, scope and applications

Brian H. M. Kuijpers

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Promotor: Prof. dr. F. P. J. T. Rutjes

Copromoter: Dr. F. L. van Delft

Manuscriptcommissie:
Prof. dr. O. C. Boerman
Dr. D. W. P. M. Löwik
Dr. P. J. L. M. Quaedflieg (DSM Pharmaceutical Products, Geleen)

Paranimfen:
A. (Bram) R. Keereweer
H. (Erik) A. M. Franck

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Aan mijn ouders

LIST OF ABBREVIATIONS

Ac	acetyl
AcOH	acetic acid
Bn	benzyl
Boc	*tert*-butoxycarbonyl
br	broad
Bu	butyl
Bz	benzoyl
°C	degrees Celcius (centigrade)
calcd	calculated
Cbz	benzyloxycarbonyl
d	days
d (NMR)	doublet
dba	dibenzylidene acetone
dd (NMR)	doublet of doublets
d.e.	diastereomeric excess
DCC	*N*,*N*-dicyclohexylcarbodiimide
DCM	dichloromethane
DIBAL	diisobutylaluminum hydride
DIPEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
dt (NMR)	doublet of triplets
e.e.	enantiomeric excess
e.g.	*exempli gratia* (for example)
equiv	equivalents
ESI	electrospray ionization
Et	ethyl
et al.	*et alia* (and others)
Fmoc	9-fluorenylmethoxycarbonyl
GC	gas chromatography
h	hour(s)
HOBt	*N*-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
i.e.	*id est* (that is)
IR	infrared
m (NMR)	multiplet
M	molar
MALDI	matrix-assisted laser desorption ionization
Me	methyl
MeCN	acetonitrile
min	minutes
mp	melting point
MS	mass spectrometry
m/*z*	mass to charge ratio
NBS	*N*-bromosuccinimide
NMR	nuclear magnetic resonance
o	ortho
OSu	hydroxysuccinimide
p	para
Ph	phenyl
ppm	parts per million
q	quartet
quant.	quantitatively
RCM	ring-closing metathesis
R_f	retention factor
rt	room temperature
s	singlet (NMR)
t (NMR)	triplet
TBDPS	*tert*-butyldiphenylsilyl
t-Bu	*tert*-butyl
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl
Trt	trityl (triphenylmethyl)
Ts	*p*-toluenesulfonyl

CONTENTS

The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not 'Eureka!' but 'hmm…That's funny…'

-Isaac Asimov

A good gulp of hot whiskey at bedtime
it's not very scientific, but it helps.

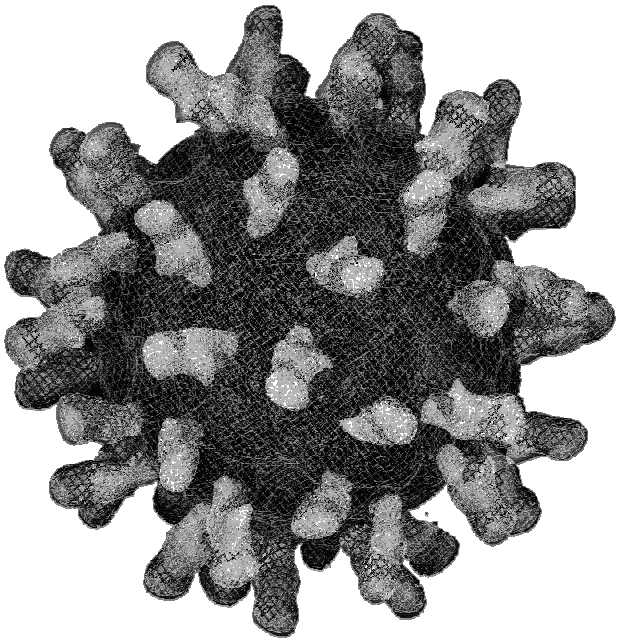
-Alexander Fleming

CHAPTER

1

Introduction

Abstract:
Naturally occurring glycosylated peptides and proteins play an important role in various biological processes, and are therefore interesting molecules for the preparation of new therapeutic drugs. However, synthesis of these natural glycopeptides is frequently hampered by the sensitivity of the glycosidic linkages to acidic and basic conditions. Another important pathway for glycopeptide degradation involves enzymatic cleavage of the carbohydrate moiety. A search for new, more stable mimics led to the development of *C*-linked isosteres, providing excellent chemical and enzymatic stability without negatively influencing the biological properties.



1.1 GENERAL INTRODUCTION

As the result of years of worldwide interest in 'genomics' and 'proteomics', our current knowledge of basic structure and function of proteins has reached a level of high maturity. In contrast, our understanding of co- and posttranslational processes that are not under direct genetic control, *e.g.* phosphorylation, glycosylation, sulfation or lipidation, is rather poor. Posttranslational protein modification, however, often has a dramatic influence on protein complexity and exerts control over vital biological functions.[1] For example, dynamic phosphorylation of proteins plays a decisive role in regulation of enzyme activity, which has *inter alia* led to enormous interest in kinase inhibitors for cancer treatment.[2] Even less developed is our understanding of protein modification by glycosylation, despite the fact that as many as 50% of human proteins are *O*- or *N*-glycosylated.[3]

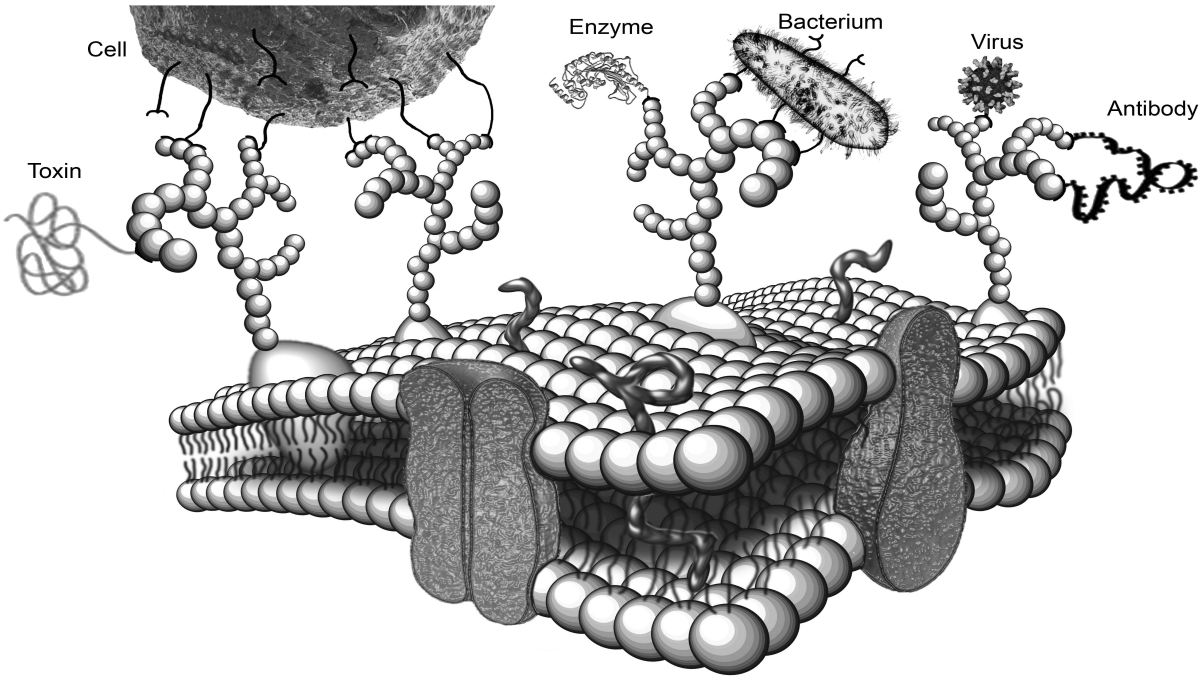


Figure 1. *Schematic representation of interactions of cell-surface glycoproteins.*

Protein glycosylation plays a crucial role in correct protein folding and thermal and proteolytic stability. As a consequence, errors in protein glycosylation cause various diseases,[4] such as autoimmune diseases, storage diseases, diabetes and cancer. Furthermore, cell surface or membrane-bound glycoproteins (Figure 1) are essential mediators in cellular processes[5] such as cell growth, cell differentiation, cell-cell

adhesion and intercellular communication. The essential connection in any glycoprotein is a covalent linkage between an anomeric center of a mono- or oligosaccharide and an amino acid side-chain of a protein. Typically, glycoproteins are categorized based upon the bond connecting the carbohydrate moiety to the amino acid functionality. Most commonly, *N*-glycoproteins contain an amide linkage to asparagine while the carbohydrates of the *O*-glycoproteins are bound to serine or threonine via an ether bridge.[6] Less common are *N*-glycoproteins via the arginine side-chain and *O*-glycosidic linkages to hydroxylysine, 4-hydroxyproline and tyrosine. A few *C*-glycoproteins, *S*-glycoproteins and *P*-glycoproteins are also known, but not covered here.[7] Despite the enormous diversity of glycoproteins known, only eight simple carbohydrates are involved in protein glycosylation, *i.e.* D-glucose,D-galactose, D-mannose, L-fucose, D-*N*-acetylneuraminic acid (sialic acid), D-*N*-acetylgalactosamine, D-*N*-acetylglucosamine, and D-xylose.[7] Therefore, the unique structure of glycoproteins is not so much determined by the nature, but primarily by the arrangement of the sugar constituents. Since each carbohydrate has multiple hydroxy groups available for substitution, over 35,560 different tetrasaccharides can be generated from only four different sugars.[8]

The *N*-linked glycoproteins are the most abundant in Nature[3] and are commonly divided into three groups: high-mannose, complex, hybrid and poly-*N*-acetyllactosamine glycans. All *N*-glycans share a common pentasaccharide core structure $Man_3GlcNAc_2$[9] as shown in Figure 2. The pentasaccharide core can be extended by up to five antennae, with high structural diversity in terms of substitution pattern, degree of branching and terminal sugars.

The *O*-linked glycoproteins or *O*-glycans, second in order of biological importance, show a much higher degree of structural diversity, and eight core structures have been found[10] (Figure 2). The majority of the *O*-linked chains on serum and membrane glycoproteins are of the sialylated tri- and tetrasaccharide type based on type 1 core, characterized by sialic acid α-2,3-linked to galactose and/or α-2,6-linked to GalNAc. Longer and more diverse oligosaccharides, *e.g.* incorporating L-fucose or KDO, have also been detected. Another type of *O*-glycosylation, not discussed here, takes place on nuclear and cytoplasmic proteins. In complex interplay with the well-studied protein *O*-phosphorylation/dephosphorylation, protein GlcNAc-ylation modulates signaling, and influences protein expression, degradation and trafficking.[11]

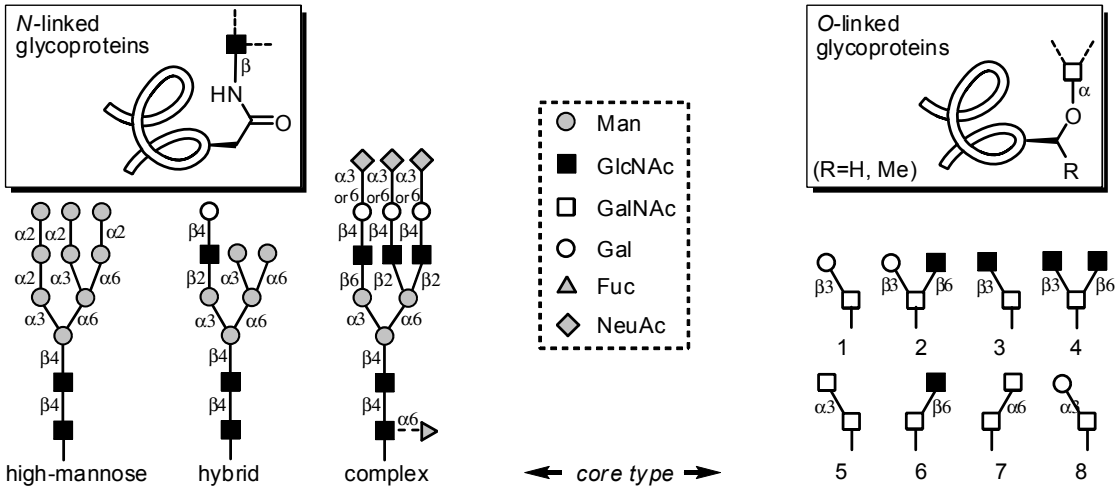


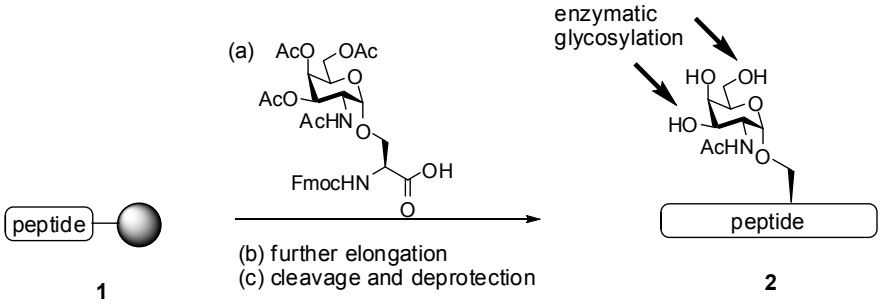
Figure 2. *Core structures of natural N- and O-linked glycopeptides. Symbols adopted from the Consortium of Functional Glycomics.*[12]

The importance of protein glycosylation has provided a strong stimulus for research into glycoprotein glycobiology and applications for therapeutic approaches.[13] Unfortunately glycoproteins, both *N*- and *O*-linked, are typically expressed as mixtures of heterogeneous glycoforms that possess the same peptide backbone but differ in both the nature and site of glycosylation. As a consequence, the difficulty of obtaining homogeneous samples has significantly hampered our understanding of glycoprotein structure and function.[14] In practice, glycosylation analysis, *e.g.* with HPLC or mass spectrometric techniques, is performed on a complex mixture of glycoforms, which complicates full structural and functional characterization. By the same token, heterogeneity in protein expression is also a significant hurdle in large-scale biotechnological production of glycoproteins.

1.2 SYNTHESIS OF GLYCOPEPTIDES AND PROTEINS

The difficulty to obtain pure glycoproteins from natural sources has sparked wide interest in the production of glycoproteins by alternative means.[14,15] Natural glycopeptides can serve as new therapeutic drugs,[15,16] *e.g.* against carbohydrate-based metabolic disorders,[17] and in the battle against cancer[18] and AIDS.[19] In addition, non-natural glycosylation of peptide-based drugs may enhance their solubility, resistance against enzymatic degradation, and can positively influence biological and pharmacokinetic properties.[20]

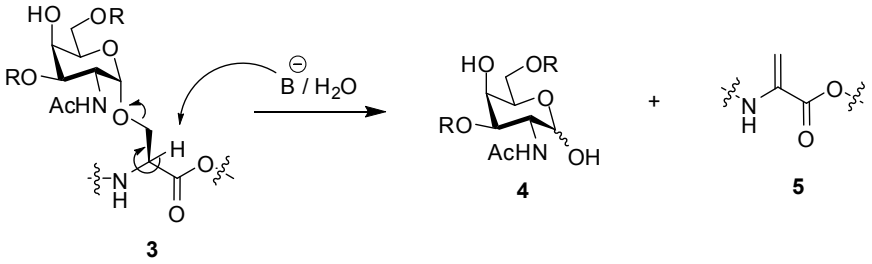
However, at present only few glycopeptides or proteins are in use as treatment against diseases.[6,21] Firstly, as delineated above, biotechnological production of glycoproteins inevitably leads to mixtures of glycoforms, as for example is the case for α-erythropoietin (EPO).[22] Unfortunately, the large size of glycoproteins hampers a straightforward preparation by synthetic means, necessitating advanced molecular biology tools or a combination of protein expression systems, biochemical techniques and chemical modification.[15] Smaller glycopeptides, however, typically of sizes up to 50 amino acids, offer the possibility of synthetic preparation in homogeneous form by a range of techniques.[23] For example, a large number of *O*-linked glycopeptides for cancer vaccination purposes have been prepared by incorporation of a presynthesized *O*-glycosylserine building block making use of standard solid-phase peptide synthesis[24] (Scheme 1).



Scheme 1. *Solid-phase peptide synthesis towards* O*-linked glycopeptides.*

However, several problems may arise when applying glycoamino acid building blocks. Firstly, such an approach is suitable only for relatively small glyco-fragments, since steric hindrance leads to impractically low coupling yields for larger oligosaccharides. Similar arguments apply to *N*-linked glycoamino acids.

Apart from that, reaction conditions required for final deprotection steps of *O*-linked glycopeptides must be carefully tuned in order to prevent undesired β-elimination[25] (Scheme 2), whereas acidic conditions may lead to cleavage of the rather unstable fucosyl fragments, if present. Hence, a large number of groups have focused on chemoenzymatic approaches for synthesis of complex glycoproteins, involving incorporation of a single sugar by chemical synthesis, followed by enzymatic elaboration after final deprotection of the glycopeptides. In this fashion, complicated glycopeptides have been assembled, but clearly such a strategy is limited by access to the requisite glycosyltransferases.



Scheme 2. *β-Elimination process.*

Apart from (chemo)enzymatic procedures, purely enzymatic approaches have also been explored. The mild conditions of biocatalytic peptide bond formation avoid the potential pitfalls associated with chemical synthesis as discussed above. An additional advantage is the lack of racemization, while at the same time side-chain protecting groups can mostly be omitted. Unfortunately, a general method for chemoenzymatic synthesis of peptides has not been achieved yet, with major limitations being the hydrolytic activity of the protease and the enzyme specificity. Moreover, enzyme specificity limits the choice of suitable amino acids for synthesis of a peptide bond. Finally, glycopeptides have also been prepared by expression in eukaryotic systems,[26] however it is not uncommon for them to exhibit glycoform microheterogeneity.[27]

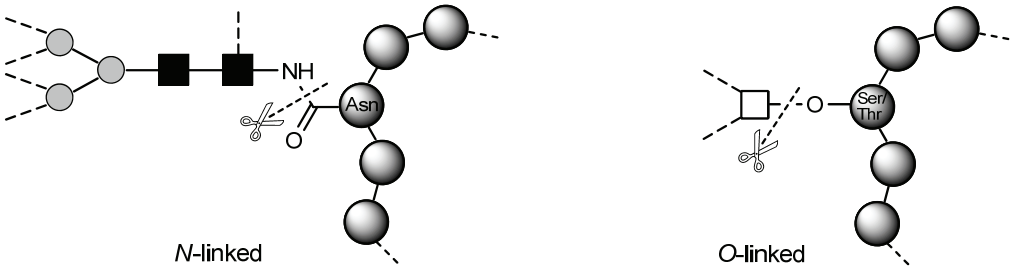


Figure 3. *Example of the actions of naturally present endoglycosidases.*[30]

It is well-known that carbohydrate fragments on proteins substantially extend the protein lifetime under physiological conditions by offering protection against proteolytic degradation. However, the glycopeptide linkage itself may be a target of enzymatic cleavage of the carbohydrate moiety from the peptide backbone. For instance, GlcNAc-β-Ser/Thr can be cleaved by the action of β-*N*-acetylglucoamidase,[28] an enzyme expressed in every human tissue.[7a] Likewise, several (commercially available) endoglycosidases and glycosidases, originated from

bacteria and plants, have been employed to cleave the naturally occurring glycosidic *N*- and *O*-bonds (Figure 3).[29]

1.3 ISOSTERES OF THE NATURAL *N*- OR *O*-LINKED GLYCOSIDIC AMINO ACIDS

The drawbacks encountered in preparing natural glycopeptides, the sensitivity of the glycosidic bond and the disadvantages of non-chemical approaches, led to the search for metabolically stable derivatives of the naturally occurring *O*- and *N*-linked glycosides. Not only should these new unnatural glycosidic amino acids increase the chemical and metabolic stability, additionally they should not negatively alter the conformation of the glycoprotein, in other words they must truly behave as isosteres. In this regard, ether bonds (*O*-linked) have been mimicked by replacement of the oxygen with sulfur or carbon to form *S*-linked and *C*-linked isosteres **6** and **8**, repectively (Figure 4).

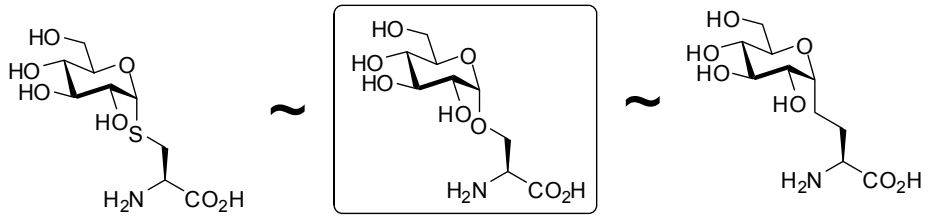


Figure 4. *Synthetic S- and C-linked glycopeptides.*

Synthetic examples of *S*-[31] and *C*-linked glycopeptides[7,32-37] (**6** and **8,** Figure 4), exhibited virtually the same conformation as the natural *N*- and *O*-linked glycopeptides[38] besides being chemically as well as metabolically more stable. For instance, the group of Bertozzi[39] showed that *C*-linked glycosides are robust and are known to sustain glycosidase degradation, glycosyltransferases, acid hydrolysis, as well as anomeric acetal- and β-elimination. In addition, solution conformations similar to the native conformation[40] and biological activities similar to the native counterparts have been observed. Further investigation by the Bertozzi group,[41] including incorporation of a *C*-linked (*S*)-serine analogue into a peptide, led to the conclusion that these structures were perfectly suited for incorporation into drugs as stable *O*-linked serine isosteres. The latter assumption was corroborated by synthesis

of a *C*-linked galactosphingolipid analogue that blocked the interaction of recombinant HIV-1 gp120 with GalCer, and had an IC_{50} value comparable to the natural sphingolipid.

Contrary to *O*-linked mimics, analogues of natural amide (*N*-linked) bonds are scarcer. The efforts of some groups to synthesize metabolically stable analogues of the *N*-linked glycosidic bonds are depicted in Figure 5. Besides the ethylene isostere **11** prepared by several groups, Kessler *et al.*[42] reported the synthesis of the retro amide **9** derived directly from the natural amide by reverting the orientation of the bond, resulting in a stable *C*-glycosidic analogue.

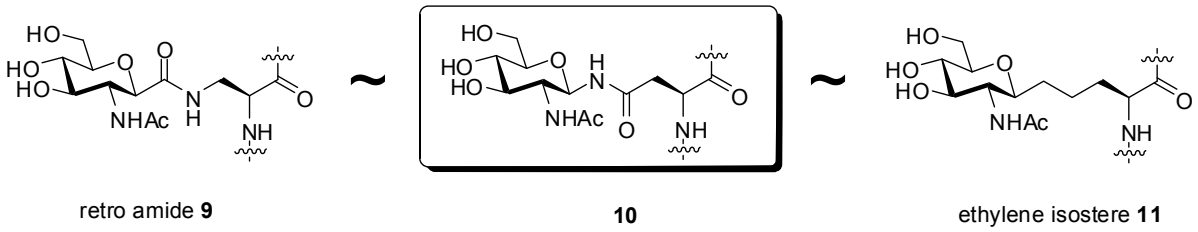


Figure 5. *Natural N-linked glycopeptide **10**, the retro amide **9** and the C-glycosyl analogue **11**.*

In addition to *C*-glycosidic amino acids closely resembling *O*- and *N*- glycopeptides, different *C*-linked building blocks have been prepared as well (e.g. *C*-glycosyl alanines, *C*-glycosyl tyrosines, *C*-glycosyl tryptophans and various miscellaneous compounds, Figure 6).

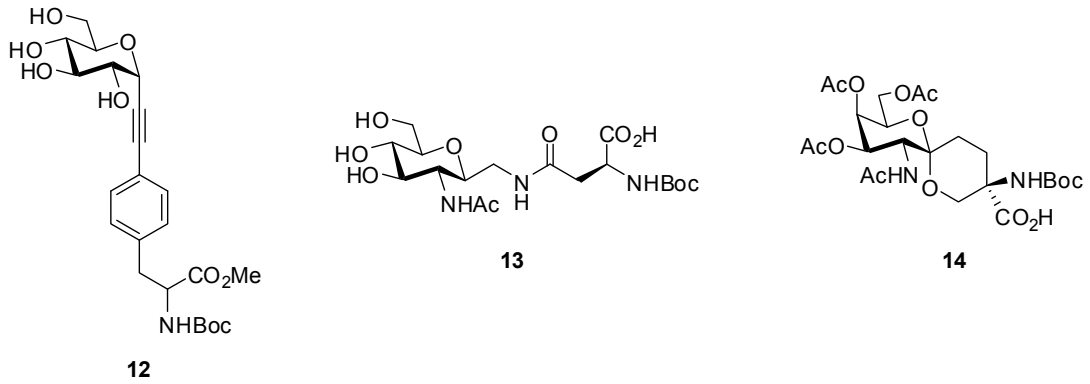


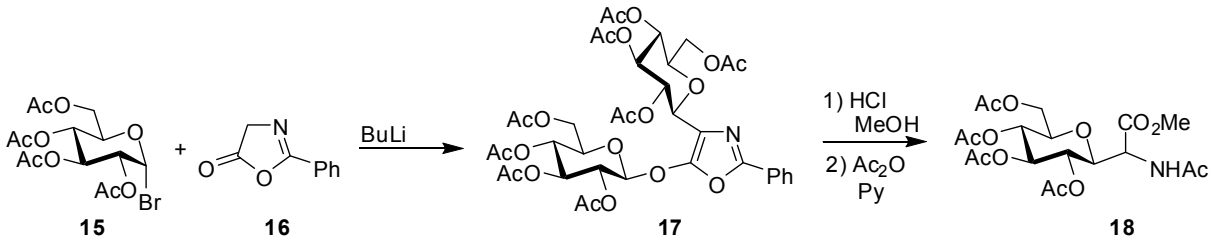
Figure 6. *Selected examples of miscellaneous C-glycosidic amino acids.*

Examples of the latter are the glycosylacetylenephenylalanine derivative **12**[43] and the conformationally constrained *O*-glycosyl serine **14**,[34] while Lee *et al.* described the synthesis of a *C*-linked glycopeptide **13** by inserting an additional carbon between the natural amide bond and the glycoside.[44] A high-mannose derivative of such a compound was found to be resistant to *N*-glycanase-catalyzed hydrolysis while displaying inhibitory activity toward the enzyme.

Due to their superior metabolic and chemical stability without negatively influencing the pharmaceutical properties, carbon analogues of *N*- and *O*-linked glycosides and glycoconjugates appear more and more in drug development today.[45]

1.4 PREPARATION OF C-LINKED GLYCOSIDIC AMINO ACIDS

Up until today nearly the whole repertoire of C-C ligation reactions have been applied. The first report concerning the synthesis of glucosyl and lactosyl glycines dates back from 1949, however, later attempts to repeat the procedure failed. The first well-documented synthesis, originating from 1975, was reported by Rosenthal and Brink,[46] and describes the coupling of tetra-*O*-acetyl α-bromoglucose **15** with oxazoline **16** under strongly basic conditions (Scheme 3). The yield of the bis-glycosylated oxazole **17** was rather low, but saponification led to the desired 2-(*β*-D-glucopyranosyl)glycinate **18** as a mixture of diastereoisomers.

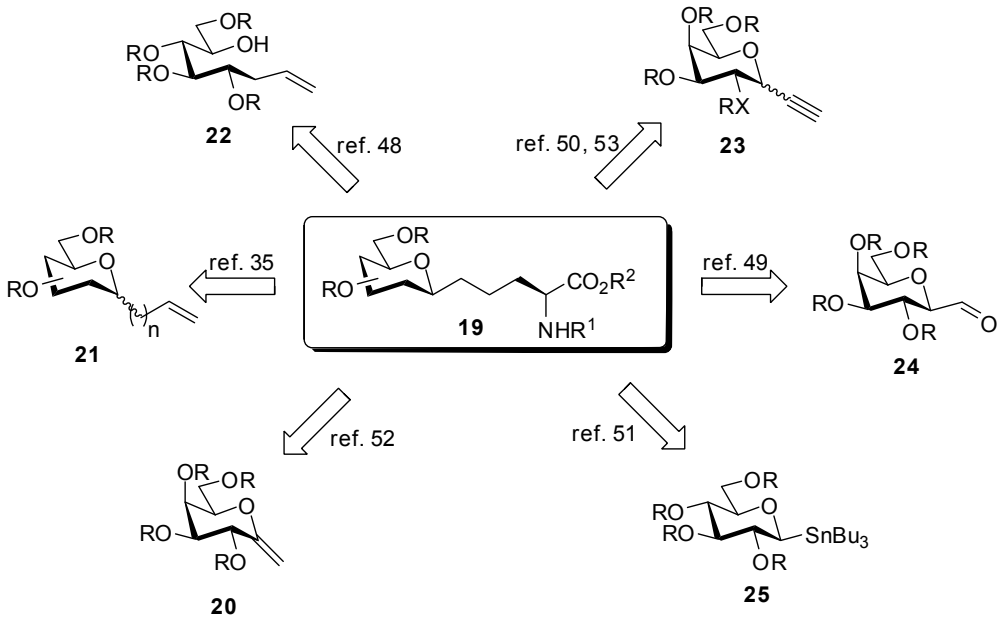


Scheme 3. *The first well-documented synthesis of a C-linked glycosidic amino acid.*

Remarkably, in the following decades only few reports on the synthesis of *C*-linked glycosidic amino acids were published, but then in the 90s due to the interest of glycosylation in pharmaceutical products suddenly many pathways were developed. Most of these methods have been extensively reviewed by the groups of Dondoni[32]and Wittmann,[47]of which the most relevant ones will be discussed below.

1.4.1 Preparation of ethylene isosteres of N-glycosyl asparagines

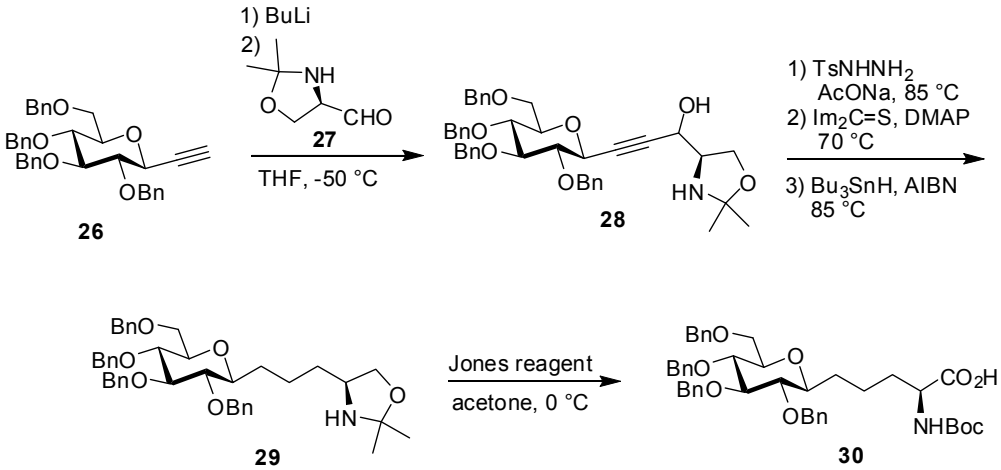
C-Linked isosteres of naturally occurring *N*-glycosyl asparagines have been prepared *via* multiple strategies as depicted in Scheme 4. These pathways include various cross-metathesis approaches[35,48] (described in Chapter 2), as well as a Wittig,[49] and an alkyne coupling strategy,[50] the latter being reported by the group of Dondoni. Moreover, Kessler *et al.*[51] described the first synthesis of an asparagine analogue in 1997 starting from the tributyltin derivative **25**. A Suzuki coupling strategy was reported for compound **20** by Taylor *et al.*[52]



Scheme 4. *Synthesis of N-glycosyl asparagine analogues from a diversity of starting materials.*

Among these approaches the metathesis approaches from Nolen *et al.,* discussed in Chapter 2, provided the highest overall yields. However, another noteworthy method was described in 2002 by Dondoni *et al.*[53] and is considered a straightforward route for the synthesis of ethylene isosteres of *N*-glycosyl asparagines **19**. Coupling of the lithium acetylide derived from **26** with Garner's aldehyde led to alkyne **28** (Scheme 5). Subsequently, diimine reduction of the alkyne, followed by Barton-McCombie deoxygenation of the hydroxyl group afforded *C*-glycoside **29**. A final oxidative cleavage of the oxazolidine ring with Jones reagent yielded the ethylene-linked analogue of *N*-glycosyl asparagine (**30**). Besides the depicted acetylated glucose, a variety of other sugars could be ligated applying this

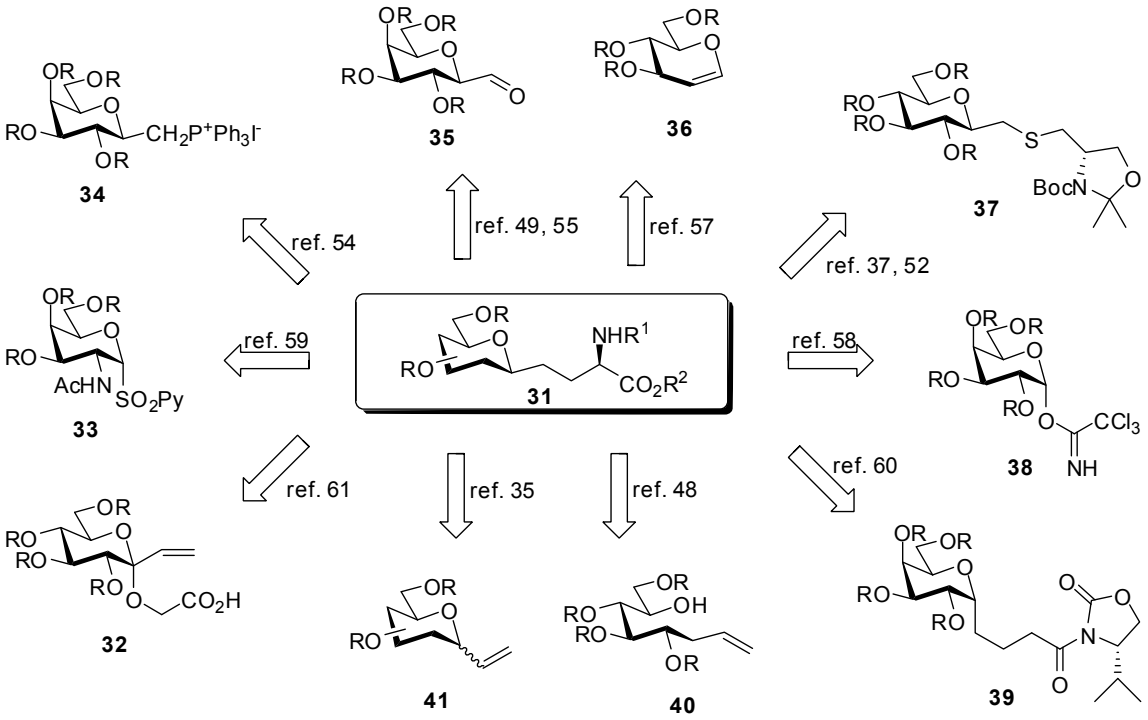
procedure, comprising the gluco, galacto, manno, and the corresponding 2-acetamido residues synthesizing both α- and *β*-anomers. This approach meets both important criteria for an excellent synthesis, namely a) it represents an easy and broadly applicable approach to a large variety of products with different configurations in the glycosidic and amino acid moieties, and b) orthogonal protecting group strategies are possible.



Scheme 5. *Synthesis of ethylene isosteres of N-glycosyl asparagine by Dondoni et al.*

1.4.2 Preparation of *O*-glycosyl serine analogues

Besides several routes applicable for the formation of *N*-asparagine isosteres (*e.g.* Wittig starting from the carbohydrate aldehyde,[49] cross metathesis[35,48]) many pathways were developed for the preparation of serine isosteres (Scheme 6). These include a Wittig approach starting from the carbohydrate ylide,[54] synthesis *via* a Ramberg-Bäcklund rearrangement,[37,52] a Wittig-Horner olefination followed by catalytic asymmetric hydrogenation,[55,56] an organozinc-glucal coupling,[57] a Lewis-acid-promoted coupling of galactopyranosyl trichloroacetimidate **38,**[58] reductive samariation,[59] electrophilic amination of *C*-glycosyl oxazolidinone enolates[60] and an anionic [2,3]-Wittig sigmatropic rearrangement of ethanoyl D-vinylketoglucoside **32**.[61]

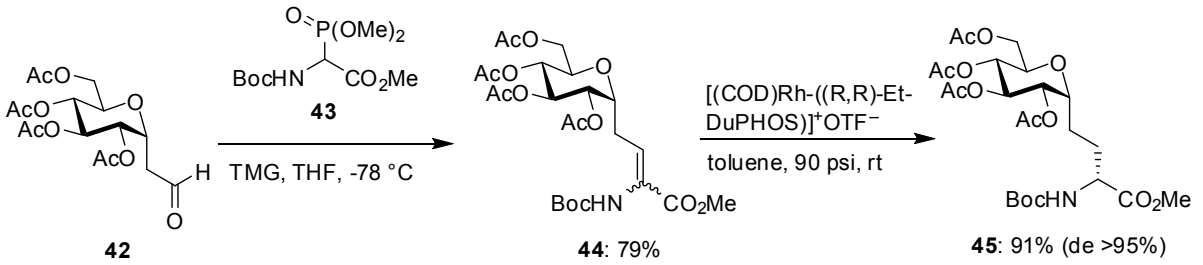


Scheme 6. *Retrosynthesis of O-glycosyl serine analogues.*

Whereas the cross metathesis approaches were high yielding for the asparagine analogues, serine derivatives could only be obtained in low yields. The shortest and highest yielding route to *C*-linked serine analogues applied the Wittig-Horner olefination followed by asymmetric hydrogenation as reported in 1997 by Toone *et al.*[55]

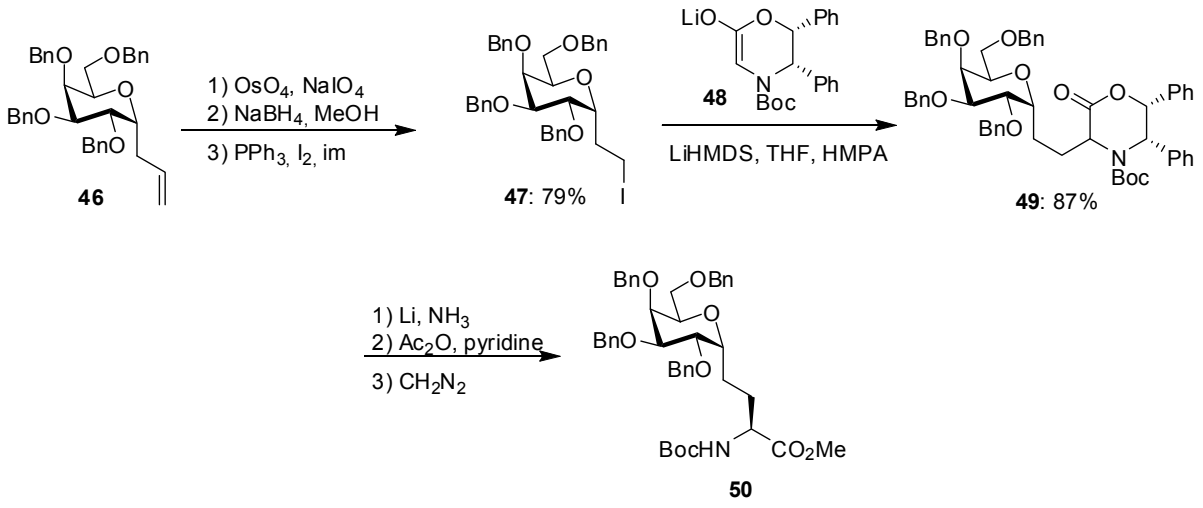
The latter approach starts with the olefination of the Boc-protected phosphonate methyl ester **43** and carbohydrate **42** *via* a Horner-Emmons reaction resulting in the formation of enamide **44** in 79% yield (Scheme 7). Subsequent hydrogenation with (*R,R*)-Et-DuPHOS-Rh$^+$ as catalyst precursor in toluene afforded the desired *C*-linked (*S*)-glycosidic amino acid **45** in good yield and with a diastereomeric excess (d.e.) of >95%. Besides glucosylated derivatives, the corresponding galacto- and manno-derived glycosidic amino acids could be prepared as well, applying slightly modified conditions (solvents, catalyst). In addition, D- and L-amino acids were accessible by changing the DuPHOS ligand (*e.g.*(*R,R*)-Et ligands resulted in (*R*)-configured products while (*S,S*)-Me ligands provided the (*S*)-isomer). The versatility of the method was extended to β-configured anomers of the glucose, galactose and mannose serine isosteres, with the obtained yields and d.e.'s being equal to those of

the α-anomers. In addition, this strategy was applied in the preparation of *β*-carbon-linked serine analogues of the Pk trisaccharide, a short peptide able to bind subunits of the Shiga-like toxin.[56]



Scheme 7. *Toone's asymmetric hydrogenation strategy.*

Another noteworthy approach reported by Nolen *et al*.[36] led to the desired product by ligation of a chiral glycine enolate derivative to a sugar iodoethyl derivative (Scheme 8). First, treatment of allylglycoside **46** with a catalytic amount of osmium tetroxide and sodium periodate provided the aldehyde, subsequent reduction with $NaBH_4$ in methanol followed by iodination (triphenylphosphine, imidazole, and iodine) afforded the desired iodoethyl carbohydrate **47**. The key step in the approach involved subjection of iodide **47** to LiHMDS, oxazinone **48**, and HMPA to afford the C-C coupled product **49**, followed by treatment with lithium in ammonia to remove the oxazinone chiral auxiliary and deprotect the benzyl ethers.



Scheme 8. *Nolen's chiral glycine enolate approach.*

A workup involving acetic anhydride followed by diazomethane treatment yielded the *C*-glycosidic product **50**. It was demonstrated that α- as well as β-anomers of gluco- and galacto-derived glycosidic amino acids could be prepared *via* the aforementioned strategy.

1.5 PURPOSE AND OUTLINE OF THE RESEARCH

The investigations described in this Thesis were part of a project to develop a set of integrated and complementary technologies for the synthesis of sugar-functionalized peptidomimetics, built from new, diverse and metabolically stable amino acid derivatives. A collaborative project between DSM (Geleen, The Netherlands), Chiralix (Nijmegen) and the Radboud University Nijmegen was funded by the agency of the Dutch Ministry of Economic Affairs (SenterNovem), supporting initiatives to stimulate sustainability and fundamental research. While DSM focused on developing new chemical and enzymatic strategies for the synthesis of linear peptides and research at Chiralix was directed at finding new strategies for the production of unnatural cyclic amino acids, it was our goal to explore new pathways for the development of stable analogues of the natural sugar amino acid linkages.

In this chapter, a general introduction to natural glycopeptides as well as an overview of recent synthetic approaches for the preparation of new glycosidic amino acid isosteres has been given.

Chapter 2 details our efforts to prepare stable glycosidic *C*-linked serine isosteres *via* a cross-metathesis reaction followed by a mercury-induced lactonization/reduction strategy.

Chapter 3 discloses a novel expedient and high-yielding approach for the synthesis of glycosidic *N*-triazole-linked amino acids, isosteres of the natural *N*-linked glycosidic amino acids.[62]

Chapter 4 describes our efforts towards a simple, stepwise coupling procedure for the preparation of glycopeptides applied in solution as well as on resin. In addition, the preparation and enzymatic resolution of the protected acetylenes are described.

In Chapter 5 a newly discovered reaction between a bromoacetylene and an organic azide coupled due to the combined action of CuI and $Cu(OAc)_2$, forming a 5-bromo trisubstituted triazole is discussed.[63]

Chapter 6 explores the possibility to apply an enzymatic coupling of *N*-triazole amino acids and amino acid amides. The results hereof are compared to coupling reactions of the natural amide-linked *N*-glycosidic amino acid.[64]

Finally, in Chapter 7 the incorporation of a *N*-triazole amino acid in a cyclic RGD peptide is described. The $\alpha_v\beta_3$-binding affinity and tumor uptake of a cyclic glycosylated RGD peptide is compared to other cyclic RGDs in *in vitro* and *in vivo* studies.[65]

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Whenever anyone says, 'theoretically,'
they really mean, 'not really.'

-Dave Parnas

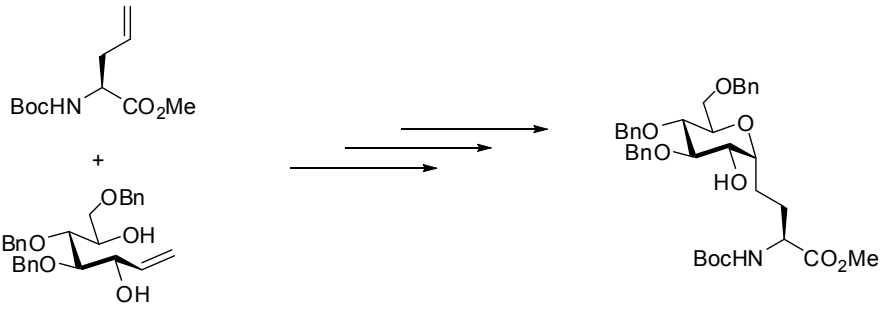
If we knew what it was we were doing,
it would not be called research, would it?

-Albert Einstein

Stereoselective Synthesis of Glycosyl Serine Methylene Isosteres by Cross Metathesis

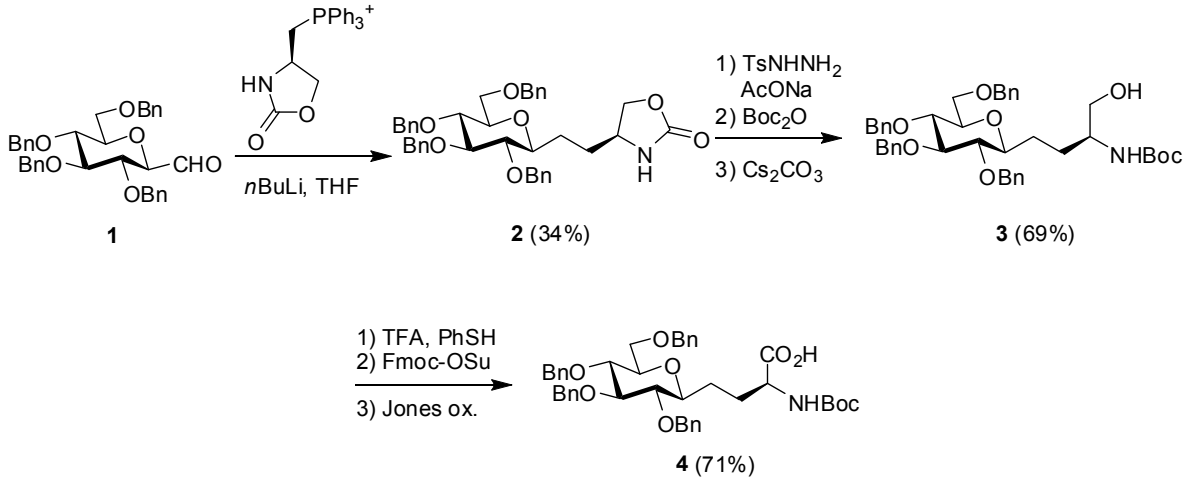
CHAPTER 2

Abstract:
C-Glycosyl amino acids mimicking the natural *O*-linked glycoamino acids, as found in glycoproteins, have been prepared *via* an olefin cross metathesis/mercury(II)-induced cyclization strategy. The resulting *C*-linked glucosyl serine analogues, obtained in a few straightforward steps, are known to surpass the natural *O*-linked analogues in stability.



2.1 INTRODUCTION

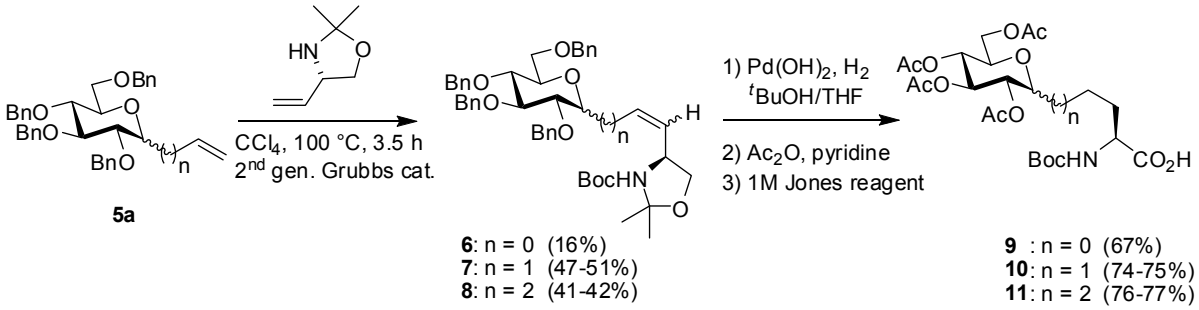
As delineated in the introductory chapter of this Thesis, there has been an extensive search throughout the years for derivatives of naturally occurring *O*- and *N*-linked glycosidic bonds with increased metabolic and chemical stability. The first known synthesis of a *C*-linked glycosyl (*S*)-serine isostere (**4**), reported in 1992 by Bednarski *et al.*[1] (Scheme 1), starts with a condensation of a serine-derived Wittig reagent with 2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl-derived aldehyde **1**.



Scheme 1. *Synthesis of the first C-linked L-serine by Bednarski et al.*

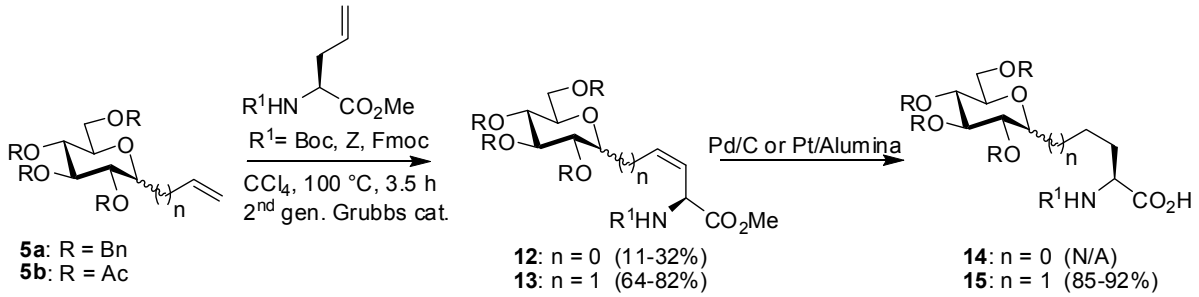
From these two advanced intermediates, the final galactosylserine isostere **4** is prepared in a seven-step reaction sequence with an overall yield of 17%. The final product **4** has the acetal oxygen of the natural linkage replaced by a stable carbon-carbon bond, an isosteric substitution with minimal difference in steric properties.

Until today several groups have explored new routes for the preparation of *C*-linked glycosyl amino acids, however most of the developed routes comprise many (low yielding) steps. One of the recent strategies that deserves attention utilizes the well-known ruthenium-catalyzed olefin cross metathesis, followed by hydrogenation and Jones oxidation as recently reported by Dondoni *et al.*,[2] thereby providing a variety of *C*-glycosyl amino acids (**9-11**, Scheme2) in moderate to good yields.



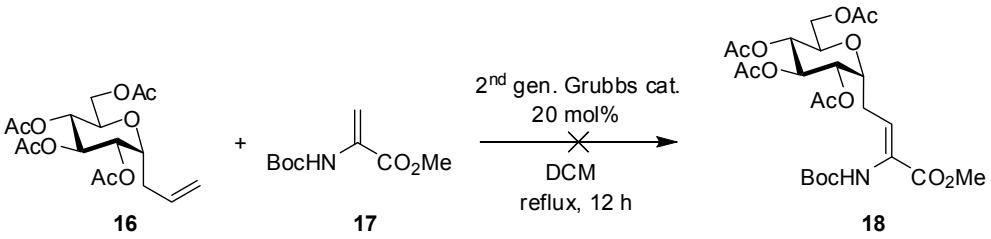
Scheme 2. *Cross metathesis route for preparation of C-glycosyl amino acids.*

Following almost the same strategy, the groups of McGarvey[3] and Nolen[4] directly coupled protected (*S*)-allylglycine (Scheme3) instead of a reduced derivative to the anomeric *C*-allyl glycosides **5** as mentioned above. Cross metathesis reactions of *C*-allyl glycosides **13** were high-yielding, but *C*-vinyl glycosides proved not to be good cross metathesis partners. Low yields of **12** were obtained ranging from 11–32%, along with recovery of high percentages of starting materials. From these studies it became clear that a cross metathesis strategy between an allylic *C*-glycoside and an unsaturated amino acid cannot lead to *C*-analogues with spacers shorter than three methylene functions, since the poor behavior of *C*-vinyl glycosides in the cross metathesis seriously diminishes the final yield.



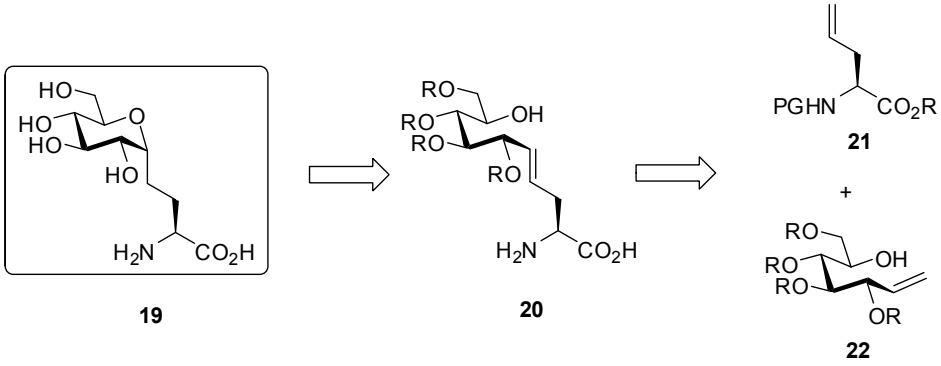
Scheme 3. *Nolen's approach for the preparation of C-glycosyl amino acids.*

Ideally, the carbon isostere of the *O*-linked glycoamino acid has the same number of connecting atoms as the natural substrate. Therefore, an alternative approach to shorten the chain between the anomeric carbon and the amino acid group conducted by the group of Nolen[4] is depicted in Scheme 4. An attempt was made to couple dehydroalanine derivative **17** to *C*-allyl glycoside **16**, however, only self-metathesis of the saccharide moiety was observed.



Scheme 4. *Alternative approach to shorten the chain length.*

We hypothesized that the poor yields of cross metathesis with a vinyl- or dehydroamino acid could be circumvented by ligation of an open-chain unsaturated sugar (*e.g.* **22**) *via* the cross metathesis strategy to an allylglycine derivative (*e.g.* **21**), producing acyclic glycoamino acid alkenes (*e.g.* **20,** Scheme 5). Subsequent cyclization was projected to lead to the desired product **19**, a mimic of the natural *O*-linked glycoamino acids with the proper number of interconnecting atoms.



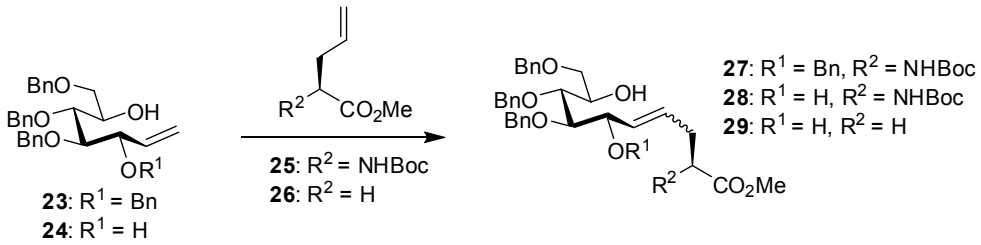
Scheme 5. *Retrosynthesis of* ***19*** *via an open-chain hydroxyalkene prepared by cross metathesis.*

2.2 C-LINKED GLYCOSIDIC SERINE ANALOGUE

To probe the feasibility of forming acyclic glycoamino acid alkenes *via* a cross metathesis approach, tetra-*O*-benzyl glucoheptenitol **23**, readily prepared by Wittig olefination of tetra-*O*-benzylglucose, and Boc-protected allylglycine methyl ester **25** were chosen as substrates for our initial experiments.

Instead of the presumed cross metathesis, only self-metathesis of the amino acid derivative occurred, producing dimer **33** (Figure 1). Given the fact that neither the homo-coupled carbohydrate derivative **31** nor the desired product **27** were formed, it appeared likely that cross metathesis is hindered by sterical hindrance around the alkene moiety of the saccharide.

Table 1. *Optimization of metathesis conditions.*



entry	R^1	R^2	solvent	T	catalyst[c]	product	yield
1	Bn	NHBoc	DCM	rt	G2 (10 mol%)	**27**	0%
2			toluene	rt	G2 (10 mol%)	**27**	0%
3			toluene	80 °C	G2 (10 mol%)	**27**	0%
4	H	NHBoc	DCM	rt	G2 (10 mol%)	**28**	37%
5			toluene	rt	G2 (10 mol%)	**28**	50%
6			toluene	80 °C	G2 (10 mol%)	**28**	22%
7			toluene	rt	G1 (10 mol%)	**28**	33%
8			toluene	rt	H (10 mol%)	**28**	28%
9			DCM	135 °C	G2 (10 mol%)	**28**	<15%[a]
10			toluene	40 °C	G2 (7 mol%)	**28**	<10%[b]
11	H	H	toluene	rt	G2 (7 mol%)	**29**	59%

[a]Performed in microwave, 10 min at 300 Watt. [b]Performed in microwave, 50 min at 100 Watt. [c]G2: 2nd gen. Grubbs catalyst (benzylidene[1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene] dichloro(tricyclohexylphosphine)ruthenium), G1: 1st gen. Grubbs catalyst (benzylidene-bis (tricyclohexylphosphine)dichlororuthenium), H: Hoveyda-Grubbs catalyst (1,3-bis-(2,4,6-trimethylphenyl)-2-imidazolidinylidene)dichloro(o-isopropoxyphenylmethylene)ruthenium).

Consequently, the less sterically hindered tri-*O*-benzyl-*gluco*heptenitol **24** was prepared by stereoselective chain-extension of 2,3,5-tri-*O*-benzyl-D-arabinose.[5] Gratifyingly, subjection of heptenitol **24** and **25** to the aforementioned cross metathesis conditions in DCM at rt (entry 4, Table 1) now led to 37% of the desired product **28**. By performing the reaction in toluene the yield was raised to 50% (entry 5). Unfortunately, further heating of the reaction mixture to 80 °C (entry 6) led to significant formation of the undesired by-product **30** (Figure 1) and a lower yield of the acyclic glycoamino acid alkene **28**. Moreover, subjection to different catalysts (entries 7 and 8) or conducting the reaction in a microwave (entries 9 and 10) did not lead to improved yields. In addition, all attempts yielded besides the desired cross metathesis product **28**, varying amounts of homo-coupled saccharide **32** and homo-coupled amino acid **33**, along with sugar derivative **30**. The latter compound is the

result of double bond isomerization to the enol, followed by tautomerization to the ketone and ring-closure.

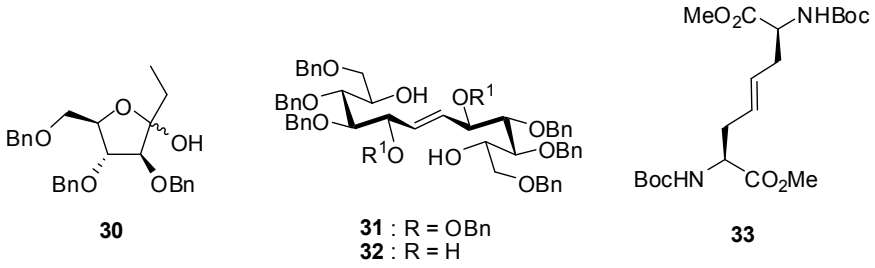
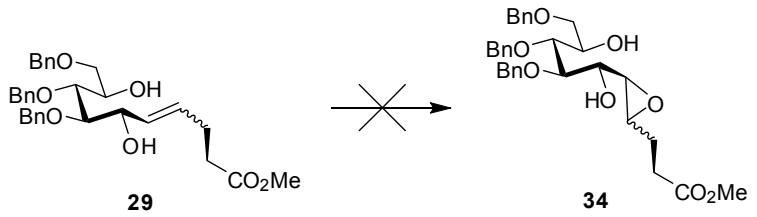


Figure 1. *By-products isolated from metathesis reactions.*

Since it appeared that the reaction could not be further optimized without lowering the overall atom efficiency, *i.e.* by using excess of one of the reaction partners, a maximum yield of 50% was an indication that reaction rates leading to the possible products are the same.[6] Finally, to examine whether the outcome of the cross metathesis is also (partially) determined by the amino acid moiety, the less sterically hindered model alkene **26** was prepared, and together with heptenitol **24** subjected to the 2nd generation Grubbs catalyst (entry 11). The result, a yield of 59% of **29**, indicates that steric hindrance on the amino acid also has some influence on the cross metathesis.

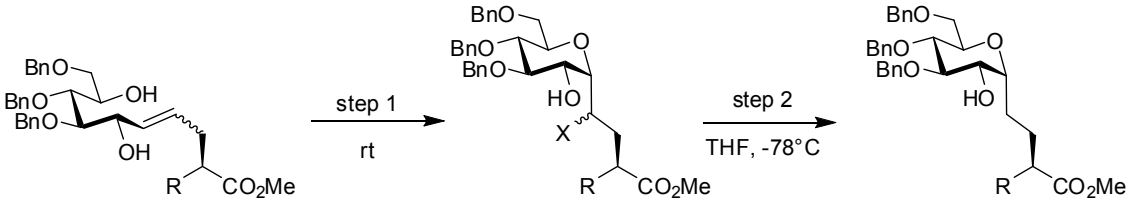
Table 2. *Epoxidation attempts of* ***29****.*



entry	conditions
1	Oxone, H_2O/MeCN, $NaHCO_3$
2	Oxone, MeOH, $NaHCO_3$
3	*m*-CPBA, DCM, $NaHCO_3$
4	$Ti(O^iPr)_4$, DCM, ^{t}BuOOH
5	Na_2EDTA, acetone, Oxone, $NaHCO_3$

Having the desired cross metathesis products in hand, an epoxidation/ring-closing strategy for the formation of the desired *C*-linked serine analogues was explored. To this end, alkene **29** lacking the α-NHBoc substituent was chosen as a model system and subjected to various epoxidation conditions (e.g. Oxone, *m*-CPBA, $Ti(O^iPr)_4/{}^tBuOOH$) as depicted in Table 2. Unexpectedly, none of the epoxidation conditions conducted led to the desired epoxide **34**, due to the fact that no transformation of **29** occurred. Several unlisted attempts to epoxidize compound **28** were also undertaken but were equally unsuccessful.

Table 3. *Direct ring-closing strategy.*



29: R = H
28: R = NHBoc

35: R = H, X = I
36: R = H, X = HgCl
37: R = NHBoc, X = I
38: R = NHBoc, X = HgCl

39: R = H
40: R = NHBoc

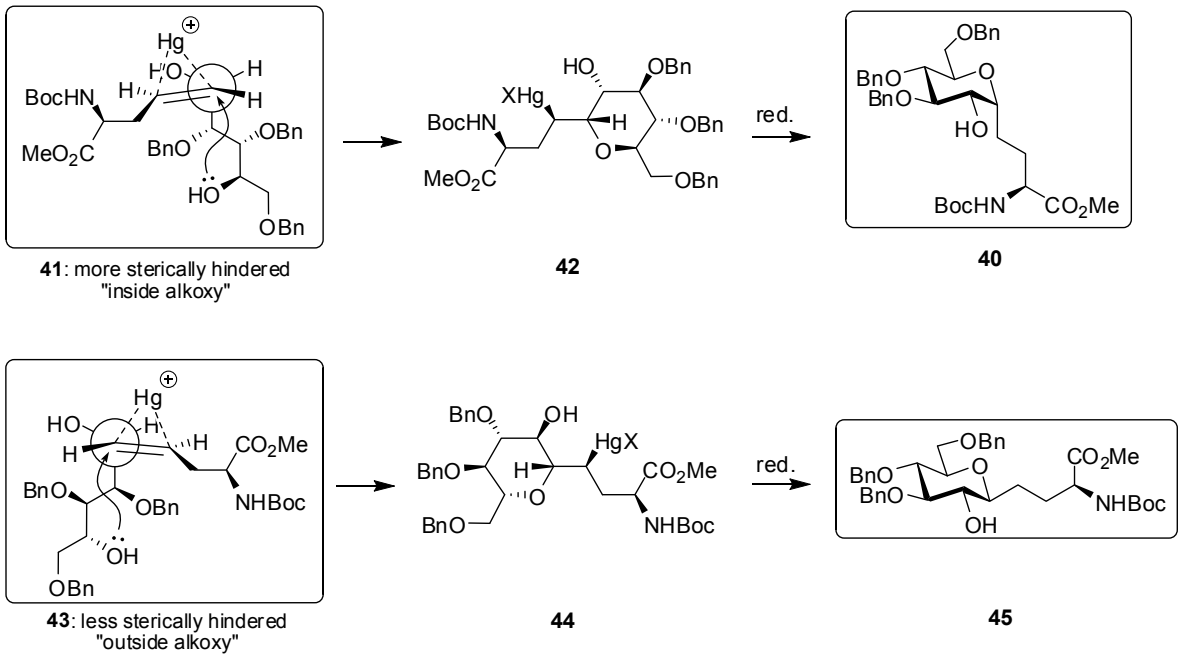
entry	R	step 1	product	yield	step 2	product	yield
1	H	IDCP, THF	**35**	0%	-	-	-
2	H	$Hg(TFA)_2$, THF	**36** [b]	85%	$LiBH_4$, Et_3B, 10 min	**39**	58%
3	NHBoc	IDCP, THF	**37**	0%	-	-	-
4	NHBoc	ICl, DCM	**37**	0%	-	-	-
5	NHBoc	$Hg(OAc)_2$, THF	**38** [b]	0%	-	-	-
6	NHBoc	$Hg(OAc)_2$, THF, dark	**38** [b]	n.d.[a]	-	-	-
7	NHBoc	$Hg(TFA)_2$, THF	**38** [b]	87%	$LiBH_4$, Et_3B, 10 min	**40**	68%
					$NaBH_4$, Et_3B, 1 h	**40**	83%

[a]Inseparable mixture of products. [b]After treatment with $Hg(OAc)_2$ or $Hg(TFA)_2$ the reactions were quenched with aqueous KCl.

Since epoxidation strategies were ineffective, we wondered whether a direct ring-closing approach, induced by electrophiles, would lead to the desired compounds (Table 3). Initial efforts to cyclize substrates **28** or **29** by subjection to a source of I^+ (IDCP or ICl), however, were without success (entries 1, 3 and 4). In addition, subjection of compound **28** to mercury acetate (entries 5 and 6), conditions known to cyclize simple heptenitols,[5,7] also failed to produce the desired ring-closed derivatives. A slight modification of the conditions, by applying the stronger

electrophile $Hg(TFA)_2$, however, smoothly afforded the desired ring-closed tetrahydropyrans **36** and **38** in yields of 85% and 87%, respectively. Subsequent reduction of the carbon-mercury bonds yielded the desired glycosidic α-linked serine analogues **39** and **40**, *via* treatment of **36** with $LiBH_4$ and **38** with $LiBH_4$ or $NaBH_4$ at −78 °C.

Stereochemistry of the ring-closed product **40** was determined by comparison of analytical data with literature reports,[8] and is in agreement with Houk's inside-alkoxy model[9] as mentioned by Nolen, were the alkyl groups favors the less hindered "anti" position, while the hydroxyl group favors the "inside" position (Scheme 6). Independently from us, the group of Nolen[8] envisioned the same strategy and recently reported their successful synthesis of a *C*-serine analogue.

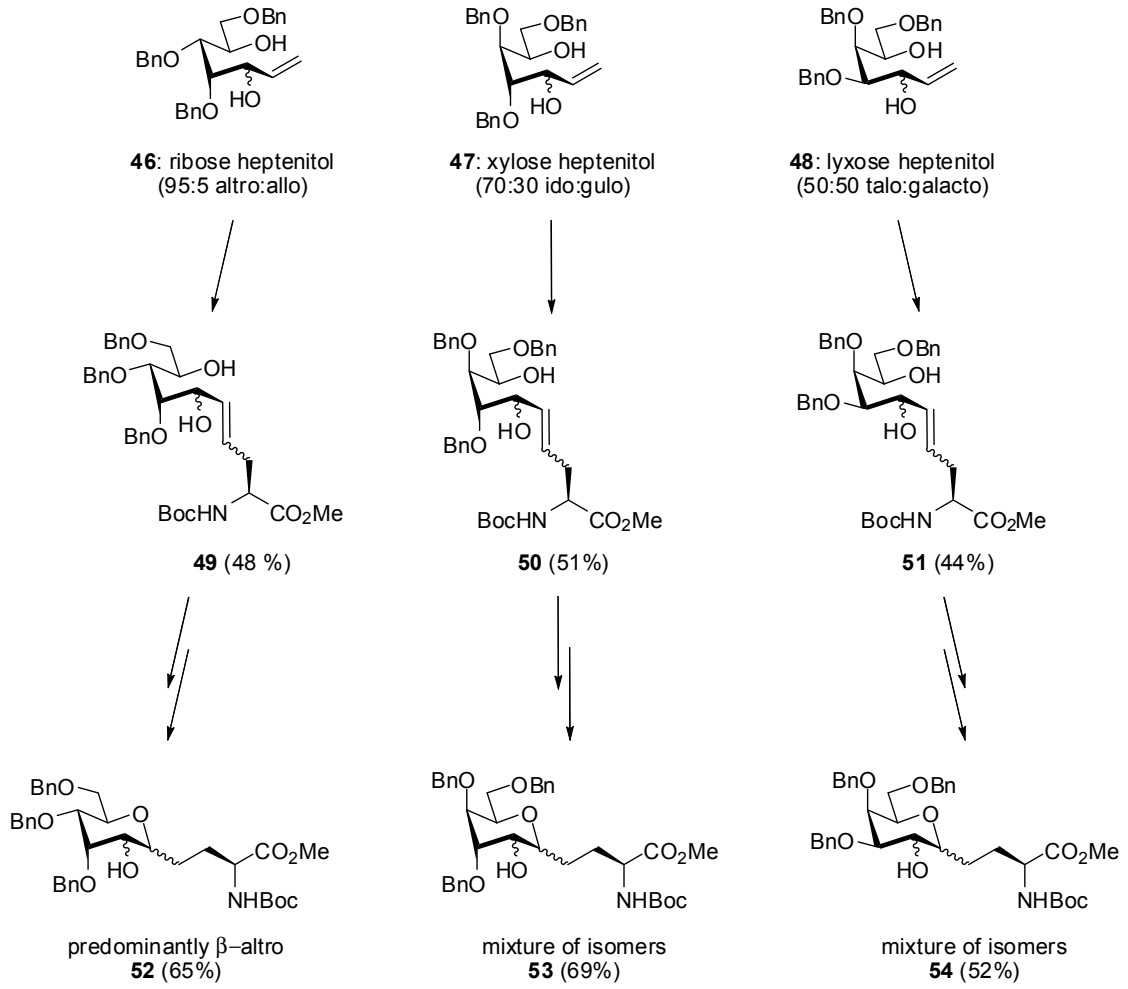


Scheme 6. *Stereochemistry rationalized using Houk's inside alkoxy model.*

2.3 VARIATION OF CARBOHYDRATE MOIETY

Having established a new route for the development of *C*-linked serine isosteres, we were intrigued if stereoisomeric sugars such as ribose, xylose and lyxose would provide the *C*-linked analogues as well. To this end, the *ribo*-, *xylo*- and *lyxo*–configured heptenitols (**46**, **47**, and **48**, respectively) were prepared in a four-step

reaction sequence, starting from the unprotected furanoses. In contrast to the vinylation of arabinose to obtain the *gluco*-configured precursor **24**, the three heptenitols now prepared were obtained as mixtures of stereoisomers at C-2: ribose afforded a 95:5 mixture of altrose:allose, xylose a 70:30 mixture of idose:gulose and lyxose did not show any stereoselectivity yielding a 50:50 mixture of talose:galactose.

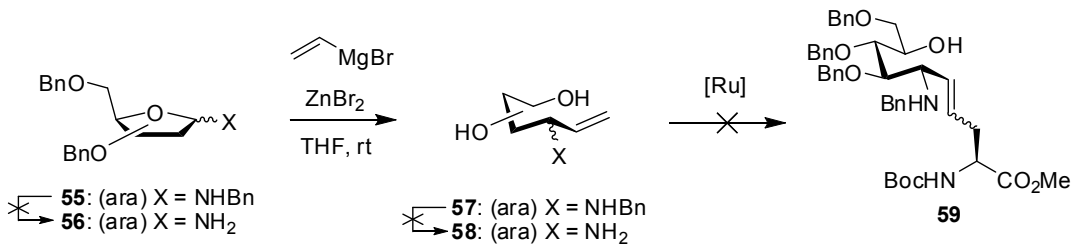


Scheme 7. *Synthesis of C-linked serine analogues from heptenitols **46-48**.*

More importantly, however, all three heptenitols **46-48** reacted smoothly with (*R*)-Boc-(*S*)-allylglycine methyl ester when subjected to 10 mol% of 2nd generation Grubbs catalyst in toluene, affording the cross metathesis products **49-51** in moderate yields (44-51%). Subsequent mercury-induced lactonization of the alkenes, followed by reduction led to the *C*-linked glycosidic serine derivatives **52-54** as mixtures of diastereoisomers, albeit with varying stereochemistry (Scheme 7).

If the stereochemistry is in accordance with Nicotra's reports and Houk's theory, the ribose saccharide led almost solely to the β-altrose derivative, although the relative configurations could not be unequivocally determined. However, for the xylose derivative **50** as well as for the lyxose derivative **51**, ring-closure followed by reduction resulted in a complex mixture of four isomers (two pairs of diastereoisomers). From these mixtures, the major isomer of the xylose product could be separated with flash-column chromatography, but also in this case the stereochemistry could not be exactly determined.

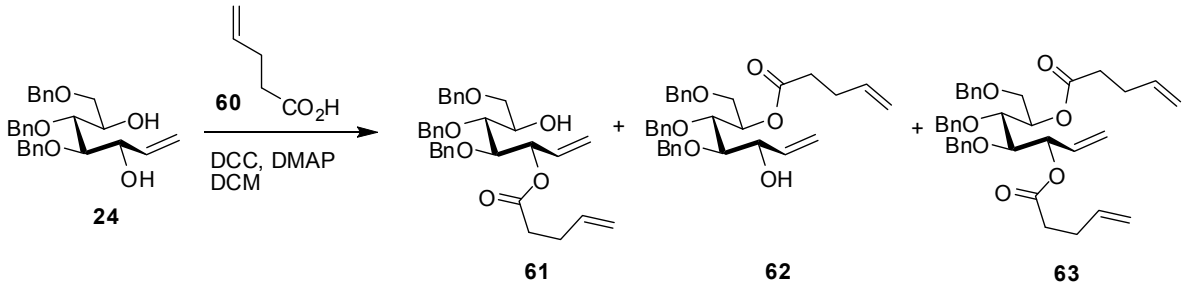
Finally, attempts were undertaken to prepare a *C*-glycosyl isostere of serine glycosylated with an 2-NHAc-glycoside. Since Nature predominantly expresses *N*-acetylgalactose as saccharide moiety attached to serine, the synthesis of such *C*-linked analogues could be of much interest. To this end, 2,3,5-tri-*O*-benzyl-D-arabinose was reacted with benzylamine to yield the anomeric *N*-benzyl derivative **55** (Scheme 8).



Scheme 8. *Attempts to prepare the glucosamine derivative* ***59****.*

Subsequent selective *N*-debenzylation was investigated under several conditions (e.g. $Pd(OH)_2$[10] or DIAD)[11] but without success. Therefore, the *N*-benzyl arabinose **55** was treated with divinylzinc, formed *in situ* from vinylmagnesium bromide and zinc bromide, to afford heptenitol **57** with complete stereoselectivity. Since steric hindrance around the amino function was expected to negatively influence the desired cross metathesis, conversion of the *N*-benzyl function to an azide appeared desirable. However, selective debenzylation of **57** again failed to produce the unprotected aminoheptenitol **58**. A single attempt to use the benzylated substrate for cross metathesis reaction with **25** was conducted. However, as expected none of the desired alkene product **59** was obtained, but only homodimer **33** was observed. These findings are in accordance with earlier observations by others that cross metathesis of an *N*-acetyl-arabino heptenitol yielded minimal amounts of product.[8]

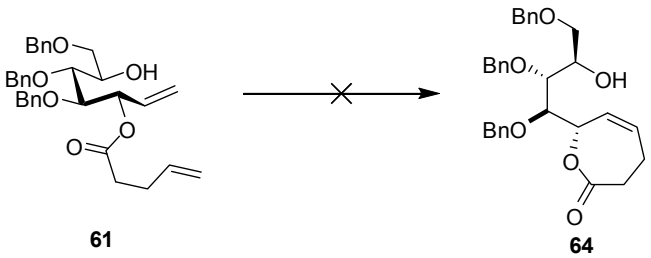
2.4 RCM



Scheme 9. *Synthesis of RCM precursor **63**.*

Although a new route for the synthesis of *C*-linked glycosidic serine analogues was successfully developed, it was reasoned that the yields of the metathesis step could be significantly improved by performing a ring-closing metathesis (RCM) instead of a cross metathesis reaction. To this end, an attempt was made for a selective esterification of arabinose heptenitol **24** with commercially available 4-pentenoic acid (**60**). However, as summarized in Scheme 9, a mixture of mono- and diesters was obtained with little selectivity for the desired ester **61**.

Table 4. *Attempts to apply the RCM approach for the synthesis of acyclic glycoamino acid alkenes.*



entry	solvent	T	catalyst[b]	product
1	DCM	rt	G1 (10 mol%)	0%
2	toluene	rt	G1 (10 mol%)	0%
3	DCM	rt	G2 (10 mol%)	mixture
4	toluene	rt	G2 (10 mol%)	mixture
5	toluene	80 °C	G2 (10 mol%)	mixture
6	toluene	135 °C	G2 (10 mol%)	mixture[a]

[a]Conducted in microwave; 10 min at 300 Watt. [b]G2: 2nd gen. Grubbs catalyst (benzylidene[1,3- bis(2,4,6-trimethylphenyl)-2- imidazolidinylidene] dichloro(tricyclohexylphosphine)ruthenium), G1: 1st gen. Grubbs catalyst (benzylidene-bis (tricyclohexylphosphine)dichlororuthenium).

More disturbingly, separation of the stereoisomers could not adequately be executed so that **61** could not be fully separated from regioisomer **62**. Nevertheless, a slightly impure sample of **61** was used to estimate the value of a RCM strategy by subjection to different metathesis catalysts as shown in Table 4. Initially, the first generation Grubbs catalyst was applied but did not provide any product (entries 1 and 2). Subjecting **61** to the 2nd generation Grubbs catalyst in all cases led to complex mixtures of inseparable compounds, with no clear major product formed.

2.5 CONCLUSION

In conclusion, a new strategy was developed for the synthesis of *C*-linked glycosidic serine analogues. Key steps of the procedure consist of a cross metathesis reaction and a mercury induced etherification. Although the cross metathesis reaction could not be executed in a yield exceeding 51%, the strategy presented here is still favored over procedures from Dondoni[2] and Nolen[4] for the preparation of stable *C*-linked serine analogues in terms of yields.

2.6 ACKNOWLEDGMENT

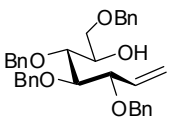
Dr. R. Blaauw (Chiralix B.V., Nijmegen, The Netherlands) and Dr. P. J. L. M. Quaedflieg (DSM Pharmaceutical Products, Geleen, The Netherlands) are acknowledged for the many fruitful discussions and advices given throughout the whole PhD period.

2.7 EXPERIMENTAL

General information.
Solvents were distilled from appropriate drying agents prior to use and stored under nitrogen. When appropriate, reactions were carried out under inert atmosphere of dry nitrogen or argon. Standard syringe techniques were applied for the transfer of dry solvents and air- or moisture-sensitive reagents. Reactions were followed and R_f values were obtained using thin layer chromatography (TLC) on silica gel-coated plates (Merck 60 F254) with the indicated solvent mixture. Detection was performed with UV-light, and/or by charring at ~150 °C after dipping into a solution of either 2% anisaldehyde in ethanol/H_2SO_4, $(NH_4)_6Mo_7O_{24}.4H_2O$ (25 g/L) or $(NH_4)_4Ce(SO_4)_4 \cdot 2H_2O$ (10 g/L) in 10% H_2SO_4. Melting points were analyzed with a Büchi melting point B-545 aparatus. IR spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer, or a Bruker Tensor 27 FTIR spectrometer. NMR spectra were recorded on a Bruker DMX 300 (300 MHz), and a Varian 400 (400 MHz)

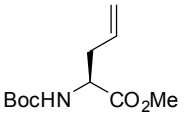
spectrometer. Chemical shifts are given in ppm with respect to tetramethylsilane (TMS) as internal standard. Coupling constants are reported as *J*-values in Hz. Column or flash chromatography was carried out using ACROS silica gel (0.035–0.070 mm, and ca 6 nm pore diameter). Optical rotations were determined with a Perkin Elmer 241 polarimeter. High resolution mass spectra were recorded on a JEOL AccuTOF (ESI), or a MAT900 (EI, CI, and ESI). MALDI-TOF-MS spectra were measured on a Bruker Biflex III machine, with dihydroxybenzoic acid (DHB) as matrix. Microwave reactions were carried out in a CEM Discover microwave.

3,4,5,7-Tetra-*O*-benzyl-D-glucohept-1-enitol (23)



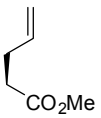
A suspension of 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (493 mg, 0.91 mmol) in dry THF (3 mL) was solubilized at 0 °C under dry nitrogen by the addition of *n*-butyllithium (0.60 mL, 1.6 M in hexanes, 0.96 mmol). A solution of methylenetriphenylphosphorane in dry THF (2 mL), prepared at −15 °C under dry argon from methyltriphenylphosphonium bromide (679 mg, 1.90 mmol) and *n*-butyllithium (1.2 mL, 1.6 M in hexanes, 1.92 mmol) was added at room temperature. Next, the mixture was refluxed for 20 min and subsequently, the excess of Wittig reagent was destroyed by the addition of acetone (3 mL). The mixture was cooled to 0 °C, filtered, and concentrated *in vacuo*. The residue was purified *via* flash column chromatography to afford **23** (328 mg, 0.61 mmol, 67%) as a colorless oil. R_f = 0.29 (EtOAc/heptanes, 1/5). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.36−7.20 (m, 20H), 5.84 (ddd, *J* = 17.1, 10.4, 7.6 Hz, 1H), 5.31−5.22 (m, 2H), 4.76 (dd, *J* = 41.0, 11.3 Hz, 2H), 4.65−4.35 (m, 6H), 4.20 (dd, *J* = 7.5, 6.1 Hz, 1H), 4.09−3.95 (m, 1H), 3.83−3.69 (m, 2H), 3.66−3.55 (m, 2H), 2.85 (d, *J* = 5.6 Hz, 1H). Spectral data are in accordance with literature.[12]

(2*S*)-*tert*-Butoxycarbonylamino-4-pentynoic acid methyl ester (25)



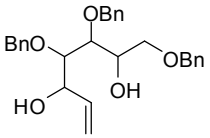
A suspension of (*S*)–allylglycine (1.13 g, 9.85 mmol) in MeOH (14 mL) was treated dropwise at 0 °C with $SOCl_2$ (0.75 mL, 10.3 mmol) and heated to reflux for 4 h. The reaction mixture was concentrated *in vacuo* to give the crude amino ester as the HCl–salt. The crude residue was suspended in CH_2Cl_2 (20 mL), Et_3N (3.0 mL, 21.6 mmol) and Boc_2O (4.2 g, 19.2 mmol) were added and the reaction mixture stirred overnight at room temperature. The mixture was concentrated *in vacuo* and purified *via* flash column chromatography to afford 25 (2.08 g, 9.07 mmol, 92%). R*f* = 0.29 (EtOAc/heptanes, 1/5). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.69 (ddt, *J* = 17.0, 9.7, 7.2 Hz, 1H), 5.15 (dd, *J* = 3.0, 1.3 Hz, 1H), 5.11 (dd, *J* = 3.0, 1.5 Hz, 1H), 5.03 (bd, *J* = 6.4 Hz, 1H), 4.40−4.35 (m, 1H), 3.74 (s, 3H), 2.58−2.43 (m, 2H), 1.44 (s, 9H). Spectral data are in accordance with literature.[13]

Methyl 4-pentenoate (26)



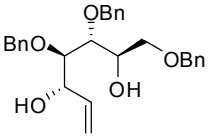
A suspension of 4-pentenoic acid (7.0 g, 69.9 mmol) in MeOH (20 ml) was treated dropwise at 0 °C with $SOCl_2$ (0.75 ml, 10.3 mmol) and heated to reflux for 4 h. The reaction mixture was concentrated *in vacuo* to afford **26** (7.95 g, quant.) as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.85−5.79 (m, 1H), 5.06 (dd, *J* = 17.2, 1.4 Hz, 1H), 5.00 (dd, *J* = 10.2, 0.8 Hz, 1H), 3.67 (s, 3H), 2.44−2.38 (m, 4H). Spectral data are in accordance with literature.[14]

General procedure A for the preparation of the heptenitols 24, 46-48.



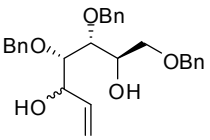
Step 1, preparation of methyl-D-furanose. To ice-cold methanol (6 mL) was added acetyl chloride (0.5 mL) and the mixture was stirred for 5 min. Subsequently the methanolic hydrogen chloride (6.5 mL) was added to a rapidly stirring solution of the unprotected D-furanose (1.0 g, 0.07 mmol) in methanol (20 mL). Stirring was continued for 3 hours at room temperature. Next, the reaction mixture was neutralized (saturated aqueous $NaHCO_3$), filtered and evaporated *in vacuo* to afford the crude methyl-D-furanose as a yellow syrup. Step 2, preparation of 2,3,5-tri-*O*-benzyl-D-methyl-furanose. Sodium hydride (60% in oil, 0.8 g) was added to a stirred solution of methyl-D-furanose (1.0 g, 6.1 mmol) in dry benzene (20 mL) and dry DMF (11 mL) at 0-5 °C, after which the mixture was stirred for 10 min. Benzyl bromide (4.2 mL) was added, and the mixture was heated till 80 °C for 2 h. Next, methanol was added to carefully decompose the excess of sodium hydrate. Subsequently the product was extracted with ether, washed with water and dried ($MgSO_4$). Purification of the product by flash column chromatography (EtOAc/Heptane 1/5) afforded 2,3,5-tri-*O*-benzyl-D-methyl-furanose. Step 3, preparation of 2,3,5-tri-O-benzyl-D-furanose. To a solution of 2,3,5-tri-*O*-benzyl-D-methylfuranose (320 mg) in acetic acid (40 mL) was added 1M H_2SO_4 (10 mL) and the mixture was heated at reflux for 2 hours. Next, the mixture was diluted with DCM (30 mL) and the organic layer was washed with water (3 × 50 mL), dried (Mg_2SO_4) and evaporated *in vacuo* to afford 2,3,5-tri-O-benzyl-D-furanose. Step 4, preparation of 1,3,4-trisbenzyloxyhept-6-ene-2,5-diol. To a solution of vinylmagnesium bromide (14.4 mL) in dry THF (1 M) a solution of dried $ZnBr_2$ (1.6 g) in THF (4 mL) was added. Subsequently, the reaction mixture was stirred for 30 min before adding a solution of 2,3,5-tri-*O*-benzyl-D-furanose (1.5 g) in THF (20 mL). The reaction mixture was quenched (saturated aqueous NH_4Cl), diluted with DCM and washed with 5% HCl, saturated $NaHCO_3$ and water. The organic layer was dried ($MgSO_4$) and evaporated *in vacuo.* Purification of the heptenitols was achieved *via* flash column chromatography using ethyl acetate-heptane mixtures.

(2*R*,3*R*,4*R*,5*S*)-1,3,4-Tris(benzyloxy)-6-heptene-2,5-diol (arabinose heptenitol) (24)



Preparation according to step 4 of the general procedure for the heptenitols starting from the 1,3,4-tris-benzyloxyhept-6-ene-2,5-diol derivative afforded **24** (1.5 g, 3.34 mmol, 93%) as a white solid. R_f = 0.62 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3425, 3084, 3062, 3023, 2863, 1079 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.37−7.22 (m , 15H), 5.91 (ddd, *J* = 17.2, 10.5, 5.6 Hz, 1H), 5.26 (ddt, *J* = 56.1, 10.5, 1.6 Hz, 2H), 4.67 (dd, *J* = 28.7, 11.2 Hz, 2H), 4.53 (d, *J* = 2.4 Hz, 2H), 4.57 (d, *J* = 14.9 Hz, 2H), 4.40 (dq, *J* = 3.5, 1.4 Hz, 1H), 4.06 (ddd, *J* = 6.8, 5.4, 3.8 Hz, 1H), 3.80−3.54 (m, 4H), 2.88 (bs, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 138.5, 138.1, 138.0, 138.0, 128.6, 128.3, 128.1, 128.1, 128.0, 127.9, 116.2, 82.0, 78.9, 74.8, 73.9, 73.6, 72.2, 71.1, 70.9. HRMS (ESI) *m/z* calculated for $C_{28}H_{33}O_5$ $(M+H)^+$: 449.2328, found: 449.2310. HRMS (ESI) *m/z* calculated for $C_{28}H_{32}O_5Na$ $(M+Na)^+$: 471.2147, found: 471.2138.

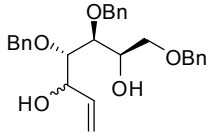
(2*R*,3*R*,4*S*)-1,3,4-Tris(benzyloxy)hept-6-ene-2,5-diol (ribose heptenitol) (46)



Preparation according to the general procedure for the heptenitols afforded **46** (1.1 g, 2.59 mmol, 73%) as a white solid. R_f = 0.62 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3468, 3084, 3058, 3028, 2859, 1082 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.37−724 (m , 15H), 5.98 (ddd, *J* =

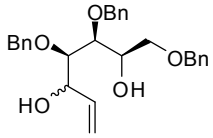
17.2, 10.6, 5.6 Hz, 1H), 5.32 (ddt, *J* = 62.6, 10.6, 1.7 Hz, 2H), 4.69−4.60 (m, 4H), 4.51 (d, *J* = 2.1 Hz, 2H), 4.45−4.43 (m, 1H), 4.11 (dt, *J* = 5.0, 4.0 Hz, 1H), 3.78 (ap t, *J* = 5.3 Hz, 1H), 3.72 (dd, *J* = 5.2, 3.7 Hz, 1H), 3.62 (bs, 1H), 3.61 (d, *J* = 2.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 138.2, 138.0, 137.9, 137.9, 128.6, 128.2, 128.1, 128.0, 128.0, 116.3, 81.0, 80.3, 74.0, 74.0, 73.6, 72.0, 71.1, 71.0. HRMS (ESI) *m/z* calculated for $C_{28}H_{33}O_5$ $(M+H)^+$: 449.2328, found: 449.2354. HRMS (ESI) *m/z* calculated for $C_{28}H_{32}O_5Na$ $(M+Na)^+$: 471.2147, found: 471.2129.

(2*R*,3*S*,4*S*)-1,3,4-Tris(benzyloxy)hept-6-ene-2,5-diol (xylose heptenitol) (47)



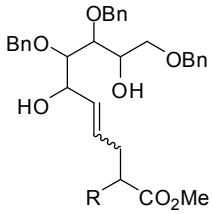
Preparation according to the general procedure for the heptenitols afforded **47** (823 mg, 1.83 mmol, 61%) as a white solid. Mixture of isomers (7:3). R_f = 0.60 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3417, 3084, 3058, 3028, 2859, 1091 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.37−7.20 (m, 15H), 5.99 & 5.91 (ddd, *J* = 17.2, 10.5, 5.8 Hz & *J* = 17.2, 10.5, 5.8, 1H), 5.31 & 5.27 (ddt, *J* = 53.4, 10.5, 1.6 Hz & *J* = 61.7, 10.6, 1.6 Hz, 2H), 4.78−4.40 (m, 7H), 4.11 & 4.06 (dt, *J* = 6.4, 1.6 Hz & *J* = 6.1, 2.9 Hz, 1H), 3.76 & 3.72 (dd, *J* = 5.1, 2.9 Hz & *J* = *6.6*, 1.7 Hz, 1H), 3.65−3.41 (m, 3H), ^{13}C NMR (75 MHz, $CDCl_3$): δ = 138.7, 138.1, 138.0, 138.0, 137.8, 137.5, 128.5, 128.4, 128.3, 128.2, 128.1, 128.1, 127.9, 127.9, 116.5, 115.6, 80.6, 80.4, 87.6, 77.3, 74.5, 74.4, 73.5, 73.4, 72.8, 71.3, 71.2, 70.7, 69.7, 68.4. HRMS (ESI) *m/z* calculated for $C_{28}H_{33}O_5$ $(M+H)^+$: 449.2328, found: 449.2336. HRMS (ESI) *m/z* calculated for $C_{28}H_{32}O_5Na$ $(M+Na)^+$: 471.2147, found: 471.2153.

(2*R*,3*S*,4*S*)-1,3,4-Tris(benzyloxy)hept-6-ene-2,5-diol (lyxose heptenitol) (48)



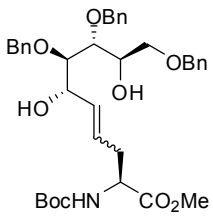
Preparation according to the general procedure for the heptenitols afforded **48** (132 mg, 0.29 mmol, 37%) as a white solid. Mixture of isomers (7:3). R_f = 0.60 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3421, 3084, 3054, 3032, 2876, 1087 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.41−7.22 (m, 15H), 6.08−5.91 (m, 1H), 5.36 & 5.26 (ddt, *J* = 31.6, 17.2, 1.7 Hz & *J* = 16.9 ,10.6, 1.6 Hz, 2H), 4.76−4.43 (m, 8H), 4.13−3.57 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 138.7, 138.0, 137.8, 133.6, 129.9, 128.6, 128.4, 128.1, 128.0, 127.8, 116.1, 116.0, 82.7, 81.2, 79.7, 79.5, 79.4, 79.1, 74.6, 74.5, 74.3, 74.0, 73.7, 73.1, 72.6, 72.4, 71.6, 71.4. HRMS (ESI) *m/z* calculated for $C_{28}H_{33}O_5$ $(M+H)^+$: 449.2328, found: 449.2366. HRMS (ESI) *m/z* calculated for $C_{28}H_{32}O_5Na$ $(M+Na)^+$: 471.2147, found: 471.2146.

General procedure B for the cross metathesis reaction.



To a solution of the saccharide (1 equiv, 0.1 M in toluene) was added a solution of the allyl substrate (1 equiv, 0.1 M in toluene) followed by the addition of the Grubbs catalyst (10 mol%). The resulting mixture was stirred at room temperature for 48 hours. Subsequently, the solvent was removed *in vacuo* and the product was isolated as a mixture of isomers *via* flash column chromatography using ethyl acetate/heptanes, 2/3.

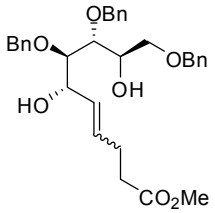
(2*S*,6*S*,7*R*,8*R*,9*R*)-Methyl 7,8,10-tris(benzyloxy)-2-(*tert*-butoxycarbonylamino)-6,9-dihydroxydec-4-enoate (28)



Preparation according to general procedure B for the cross metathesis afforded **28** (20 mg, 0.03 mmol, 50%) as a colorless oil. R_f = 0.16 (EtOAc/heptanes, 2/3). FTIR (ATR): ν = 3339, 2975, 2880, 1043 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.30−7.23 (m, 15H), 5.67−5.54 (m, 2H), 5.08

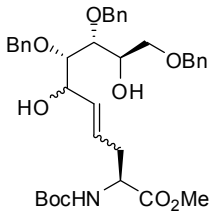
(bd, *J* = 7.8 Hz, 1H), 4.71−4.53 (m, 6H), 4.34−4.31 (m, 2H), 4.01−3.98 (m, 1H), 3.69 (s, 3H), 3.67−3.65 (m, 3H), 3.61 (t, *J* = 4.5 Hz, 1H), 3.06 (bd, *J* = 5.1 Hz, 1H), 3.01 (bd, *J* = 5.7 Hz, 1H), 2.51−2.41 (m, 2H), 1.42 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.7, 155.3, 138.2, 138.1, 138.0, 134.7, 128.6, 128.6, 128.3, 128.2, 128.1, 128.1, 128.0, 127.9, 126.0, 82.5, 80.2, 28.8, 74.7, 74.0, 73.6, 71.6, 71.1, 70.1, 53.3, 52.5, 35.4, 28.5. HRMS (ESI) calculated for $C_{37}H_{48}NO_9$ $(M+H)^+$: 650.3329, found: 650.3357. HRMS (ESI) calculated for $C_{37}H_{47}NO_9Na$ $(M+Na)^+$: 672.3149, found: 672.3140.

(6*S*,7*R*,8*R*,9*R*)-Methyl 7,8,10-tris(benzyloxy)-6,9-dihydroxydec-4-enoate (29)



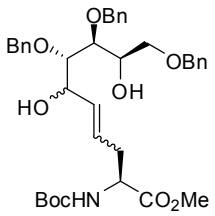
Preparation according to general procedure B for the cross metathesis afforded **29** (474 mg, 0.73 mmol, 59%) as a colorless oil. R_f = 0.47 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3447, 2915, 2863, 1731 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.38−7.23 (m, 15H), 5.73−5.65 (m, 1H), 5.53 (ap dd, *J* = 15.4, 6.3 Hz, 1H), 4.74−4.50 (m, 4H), 4.34 (bs, 1H), 4.08−4.02 (m, 1H), 3.70−3.62 (m, 6H), 3.64 (s, 3H), 2.88−2.85 (m, 2H), 2.38−2.33 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.5, 138.1, 138.0, 137.9, 131.2, 128.5, 128.3, 128.2, 128.0, 128.0, 127.9, 127.8, 82.2, 78.8, 74.7, 73.8, 73.5, 71.8, 71.1, 70.8, 51.6, 33.5, 27.6. HRMS (ESI) calculated for $C_{32}H_{38}O_7Na$ $(M+Na)^+$: 557.2515, found: 557.2530.

(2*S*,7*S*,8*R*,9*R*)-Methyl 7,8,10-tris(benzyloxy)-2-(*tert*-butoxycarbonylamino)-6,9-dihydroxy-4-decenoate (49)



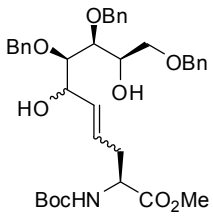
Preparation according to general procedure B for the cross metathesis afforded **49** (399 mg, 0.60 mmol, 48%) as a colorless oil. R_f = 0.16 (EtOAc/heptanes, 2/3). FTIR (ATR): ν = 3386, 2920, 2863, 1709 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.36−7.24 (m, 15H), 5.66−5.62 (m, 2H), 5.15 (bd, *J* = 7.8 Hz, 1H), 4.79−4.47 (m, 6H), 4.40−4.34 (m, 2H), 4.08−4.04 (m, 1H), 3.78−3.75 (m, 1H), 3.70 (s, 3H), 3.68−3.57 (m, 3H), 2.56−2.45 (m, 2H), 1.41 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.1, 154.9, 137.6, 137.5, 134.2, 128.2, 128.1, 128.1, 128.0, 128.0, 127.8, 127.9, 127.8, 127.8, 127.8, 127.6, 127.7, 127.6, 127.5, 127.5, 127.4, 125.8, 81.0, 80.1, 73.8, 73.7, 73.3, 71.3, 71.1, 70.4, 53.1, 52.3, 35.3, 28.4. HRMS (ESI) calculated for $C_{37}H_{47}NO_9Na$ $(M+Na)^+$: 672.3149, found: 672.3152.

(2*S*,7*S*,8*S*,9*R*)-Methyl 7,8,10-tris(benzyloxy)-2-(*tert*-butoxycarbonylamino)-6,9-dihydroxy-4-decenoate (50)



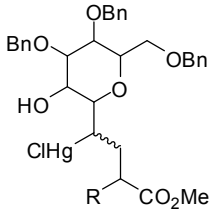
Preparation according to general procedure B for the cross metathesis afforded **50** (73 mg, 0.11 mmol, 51%) as a colorless oil. R_f = 0.41 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3417, 2924, 2859, 1740, 1709 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.36−7.22 (m, 15H), 5.71−5.54 (m, 2H), 5.29 & 5.19 & 5.08 (d, *J* = 8.1 & 8.0 & 8.0 Hz, 1H), 6.68−4.33 (m, 8H), 4.07 (bt, *J* = 5.7 Hz, 1H), 3.72 & 3.72 & 3.70 (s, 3H), 3.59−3.41 (m, 3H), 3.21 & 3.11 (bs, 1H), 2.54−2.45 (m, 2H), 1.42 & 1.41 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.7, 155.4, 138.1, 138.0, 137.9, 134.9, 128.6, 128.5, 128.4, 128.1, 128.1, 127.9, 127.7, 125.8, 80.7, 80.2, 78.3, 74.5, 74.3, 73.3, 71.1, 70.5, 68.4, 53.2, 52.4, 35.3, 28.4. HRMS (ESI) calculated for $C_{37}H_{47}NO_9Na$ $(M+Na)^+$: 672.3149, found: 672.3152.

(2*S*,7*R*,8*S*,9*R*)-Methyl 7,8,10-tris(benzyloxy)-2-(*tert*-butoxycarbonylamino)-6,9-dihydroxy-4-decenoate (51)



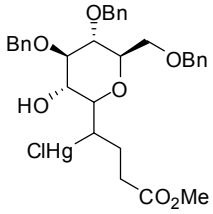
Preparation according to general procedure B for the cross metathesis afforded **51** (342 mg, 0.53 mmol, 44%) as a colorless oil. Data of mixture of isomers: R_f = 0.41 (EtOAc/heptanes, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.38−7.34 (m, 15H), 5.76−5.51 (m, 2H), 5.04 (d, *J* = 5.1 Hz, 1H), 4.76−4.51 (m, 6H), 4.41−4.30 (m, 2H), 3.09−3.67 (m, 5H), 3.70 (s, 3H), 2.59−2.33 (m, 2H), 1.42 (bs, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.6, 155.3, 138.1, 137.9, 137.7, 134.8, 134.5, 130.0, 129.9, 128.6, 128.5, 128.3, 128.2, 128.1, 128.0, 127.7, 127.6, 125.9, 82.5, 81.3, 80.1, 79.7, 79.5, 79.1, 74.4, 74.2, 74.1, 73.9, 73.7, 72.6, 71.9, 71.8, 71.1, 61.4, 60.6, 53.2, 52.4, 35.4, 35.3, 28.4. HRMS (ESI) calculated for $C_{37}H_{48}NO_9$ $(M+H)^+$: 650.3329, found: 650.3300. HRMS (ESI) calculated for $C_{37}H_{47}NO_9Na$ $(M+Na)^+$: 672.3149, found: 672.3088.

General procedure C for the mercury lactonization.



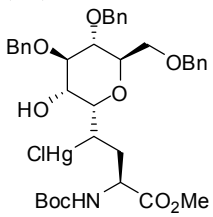
The cross metathesis product (1 equiv) dissolved in DCM (0.05 M) was added dropwise to a solution of mercury(II)trifluoroacetate (1.2 equiv) in dry DCM (0.05 M) at −78°C. After the substrate disappeared (TLC), the reaction was quenched by addition of a saturated KCl solution and stirred for 1 hour. The product was extracted with DCM (3 ×) after which the organic layer was dried ($MgSO_4$) and evaporated *in vacuo* to afford the crude mercury product as a grey solid.

(1-((3*R*,4*R*,5*R*,6*R*)-4,5-Bis(benzyloxy)-6-(benzyloxymethyl)-3-hydroxytetrahydro-2*H*-pyran-2-yl)-4-methoxy-4-oxobutyl)mercury(II) chloride (36)



Preparation according to the general procedure C for the mercury lactonization afforded **36** (125 mg, 0.162 mmol, 85%) as a grey solid. R_f = 0.67 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3421, 2924, 1722, 1264 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.38−7.19 (m, 15H), 4.71−4.46 (m, 6H), 4.20 (dd, *J* = 7.2, 1.7 Hz, 1H), 4.08 (ap dd, *J* = 9.3, 5.4 Hz, 1H), 3.83 (dd, *J* = 10.2, 6.5 Hz, 1H), 3.76−3.60 (m, 5H), 3.71 (s, 3H), 2.55−2.37 (m, 3H), 2.14−1.87 (m, 1H), 1.67 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.3, 138.1, 137.6, 137.1, 128.9, 128.8, 128.5, 128.4, 128.3, 128.0, 127.9, 127.8, 76.5, 74.4, 73.4, 73.4, 73.2, 73.0, 72.8, 67.8, 66.5, 51.9, 48.4, 35.7, 25.4. HRMS (ESI) calculated for $C_{32}H_{37}ClHgO_7Na$ $(M+Na)^+$: 793.1872, found: 793.1872.

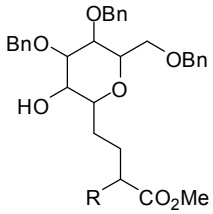
((1*R*,3*S*)-1-((2*S*,3*R*,4*R*,5*R*,6*R*)-4,5-Bis(benzyloxy)-6-(benzyloxymethyl)-3-hydroxytetrahydro-2*H*-pyran-2-yl)-3-(*tert*-butoxycarbonylamino)-4-methoxy-4-oxobutyl)mercury(II) chloride (38)



Preparation according to the general procedure C for the mercury lactonization afforded **38** (143 mg, 0.162 mmol, 87%) as a grey solid. R_f = 0.63 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3381, 3036, 2920, 2872, 1722, 1688 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.35−7.19 (m, 15H), 5.18 (bs, 1H), 4.60−4.46 (m, 6H), 4.31 (bs, 1H), 4.17 (dd, *J* = 7.0, 1.6 Hz, 1H), 4.07−4.04 (m, 1H), 3.81−3.55 (m, 6H), 3.77 (s, 3H), 2.41−1.82 (m, 2H), 1.90−1.83 (m, 1H), 1.46 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.0, 155.8, 138.1, 137.6, 137.1, 128.8, 128.6,

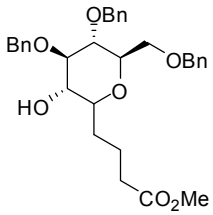
128.3, 128.0, 127.9, 127.8, 80.8, 76.3, 74.2, 73.4, 73.4, 73.3, 73.0, 72.7, 67.7, 66.3, 54.4, 52.9, 43.2, 33.0, 25.5. HRMS (ESI) calculated for $C_{37}H_{47}ClHg^{200}NO_9^{Na}$ $(M+Na)^+$: 906.2442, found: 906.2392. HRMS (ESI) calculated for $C_{37}H_{46}ClHg^{202}NO_9Na$ $(M+Na)^+$: 908.2465, found: 906.2475.

General procedure D for the reduction of the mercury substrates



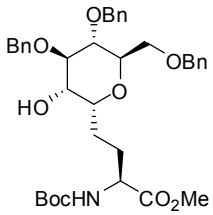
The mercury adduct (1 equiv) was dissolved in dry THF (0.2 M) and cooled to −78°C. Subsequently Et_3B (1.2 equiv) and $LiBH_4$ (1.05 equiv) were added and the mixture was stirred for 10 min at 78°C. Next, the reaction was quenched by carefully adding a solution of saturated NH_4Cl. The product was extracted with Et_2O (3 ×), and the combined organic layers were washed with water, dried ($MgSO_4$), and evaporated *in vacuo* to afford the desired glycosylated product.

Methyl 4-((3*S*,4*R*,5*R*,6*R*)-4,5-bis(benzyloxy)-6-(benzyloxymethyl)-3-hydroxytetrahydro-2*H*-pyran-2-yl)butanoate (39)



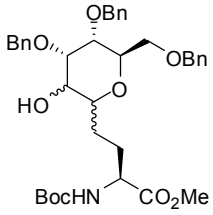
Preparation according to the general procedure D for the reduction of the mercury salt afforded **39** (24 mg, 0.04 mmol, 58%). R_f = 0.58 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3477, 3031, 2924, 2867, 1731 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.35−7.21 (m, 15H), 4.69−4.49 (m, 6H), 3.98 (ap q, *J* = 5.1 Hz, 1H), 3.89−3.85 (m, 1H), 3.81 (dd, *J* = 10.2, 5.6 Hz, 1H), 3.73−3.68 (m, 2H), 3.65 (s, 3H), 3.63 (ap t, *J* = 5.0 Hz, 2H), 2.83 (d, *J* = 7.9 Hz, 1H), 2.35 (t, *J* = 7.1 Hz, 2H), 1.84−1.55 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.1, 138.3, 138.1, 137.6, 128.7, 128.6, 128.5, 128.1, 127.9, 127.8, 127.7, 78.2, 75.3, 73.6, 73.5, 73.4, 73.1, 71.6, 69.9, 68.3, 51.6, 34.0, 27.8, 21.3. HRMS (ESI) calculated for $C_{32}H_{39}O_7$ $(M+H)^+$: 535.2696, found: 535.2686. HRMS (ESI) calculated for $C_{32}H_{38}O_7Na$ $(M+Na)^+$: 557.2515, found: 557.2496.

(S)-Methyl 4-((2*R*,3*S*,4*R*,5*R*,6*R*)-4,5-Bis(benzyloxy)-6-(benzyloxymethyl)-3-hydroxytetrahydro-2*H*-pyran-2-yl)-2-(*tert*-butoxycarbonylamino)butanoate (40)



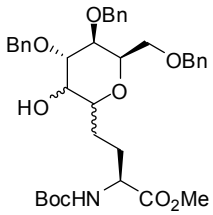
Preparation according to the general procedure D for the reduction of the mercury salt afforded **40** (30 mg, 0.05 mmol, 68%) as a colorless oil. R_f = 0.55 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3417, 2933, 1740, 1709 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.36−7.19 (m, 15H), 5.15 (d, *J* = 8.2 Hz, 1H), 4.68−4.72 (m, 6H), 4.36−4.25 (m, 1H), 3.96 (d, *J* = 5.1 Hz, 1H), 3.83 (dd, *J* = 9.9 Hz, 1H), 3.81−3.76 (m, 1H), 3.74−3.57 (m, 4H), 3.70 (s, 3H), 2.88 (d, *J* = 7.9 Hz, 1H), 2.03−1.90 (m, 1H), 1.76−1.57 (m, 3H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.0, 155.2, 138.0, 137.8, 137.3, 128.5, 128.4, 128.3, 127.8, 127.8, 127.7, 127.6, 127.5, 79.9, 78.1, 75.1, 73.6, 73.4, 73.3, 73.1, 71.7, 69.8, 68.2, 53.7, 52.4, 29.1, 28.6, 24.5. HRMS (ESI) calculated for $C_{37}H_{48}NO_9$ $(M+H)^+$: 650.3329, found: 650.3316. HRMS (ESI) calculated for $C_{37}H_{47}NO_9Na$ $(M+Na)^+$: 672.3149, found: 672.3106.

(S)-Methyl 4-((2*S*,3*R*,4*S*,5*R*,6*R*)-4,5-bis(benzyloxy)-6-(benzyloxymethyl)-3-hydroxytetrahydro-2*H*-pyran-2-yl)-2-(*tert*-butoxycarbonylamino)butanoate (52)



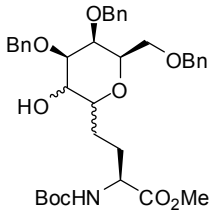
Preparation according to the general procedures C and D for the mercury lactonization followed by the reduction of the mercury salt provided **52** (133 mg, 0.20 mmol, 55% overall) as a colorless oil. Data of major compound: R_f = 0.55 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3382, 2915, 1744, 1714 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.38−7.18 (m, 15H), 5.24 (d, *J* = 8.1 Hz, 1H), 4.71 (d, *J* = 12.1 Hz, 1H), 4.60−4.49 (m, 4H), 4.41 (d, *J* = 11.5 Hz, 1H), 4.31−4.21 (m, 1H), 3.96−3.91 (m, 1H), 3.91−3.84 (m, 1H), 3.82 (d, *J* = 2.8 Hz, 1H), 3.57 (d, *J* = 2.6 Hz, 1H), 3.81−3.66 (m, 6H), 3.70 (s, 3H), 2.68−2.43 (m, 1H), 1.91−1.53 (m, 4H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.5, 154.9, 138.0, 137.7, 137.5, 127.8, 127.8, 127.8, 127.2, 127.2, 127.2, 127.1, 127.1, 127.0, 79.4, 74.8, 74.4, 74.2, 73.1, 72.6, 72.5, 71.2, 69.7, 53.2, 52.0, 28.8, 28.1, 26.3. HRMS (ESI) calculated for $C_{37}H_{48}NO_9$ $(M+H)^+$: 650.3329, found: 650.3307. HRMS (ESI) calculated for $C_{37}H_{47}NO_9Na$ $(M+Na)^+$: 672.3149, found: 672.3091.

(*S*)-Methyl 4-((4*S*,5*S*,6*R*)-4,5-Bis(benzyloxy)-6-(benzyloxymethyl)-3-hydroxytetrahydro-2*H*-pyran-2-yl)-2-(*tert*-butoxycarbonylamino)butanoate (53)



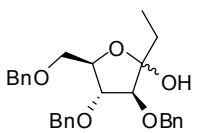
Preparation according to the general procedures C and D for the mercury lactonization followed by the reduction of the mercury salt provided **53** (31 mg, 0.05 mmol, 69%) as a colorless oil. A pure fraction of the major isomer, presumably the β-idose saccharide was isolated *via* column chromatography (11 mg, 0.02 mmol). Data of major isomer: R_f = 0.55 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3485, 2920, 2863, 1744, 1705 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.37−7.20 (m, 15H), 5.07 (d, *J* = 8.0, 1H), 4.58−4.52 (m, 4H), 4.45 (d, *J* = 11.9 Hz, 2H) 4.40 (d, *J* = 11.4 Hz, 2H), 4.31−4.24 (m, 1H), 3.99 (t, *J* = 6.5 Hz, 1H), 3.82 (t, *J* = 3.0 Hz, 1H), 3.70 (s, 3H), 3.61−3.45 (m, 4H), 3.30 (d, *J* = 11.7 Hz, 1H), 2.04−2.00 (m, 1H), 1.91−1.76 (m, 3H), 1.42 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.5, 155.6, 138.3, 137.9, 137.2, 128.7, 128.5, 128.4, 128.1, 127.9, 127.8, 79.9, 75.5, 74.5, 73.8, 73.6, 73.1, 72.5, 72.2, 69.3, 69.0, 53.5, 52.3, 289, 28.5. HRMS (ESI) calculated for $C_{37}H_{48}NO_9$ $(M+H)^+$: 650.3329, found: 650.3329. HRMS (ESI) calculated for $C_{37}H_{47}NO_9Na$ $(M+Na)^+$: 672.3149, found: 672.3134.

(*S*)-Methyl 4-((4*R*,5*S*,6*R*)-4,5-bis(benzyloxy)-6-(benzyloxymethyl)-3-hydroxytetrahydro-2*H*-pyran-2-yl)-2-(*tert*-butoxycarbonylamino)butanoate (54)



Preparation according to the general procedures C and D for the mercury lactonization followed by the reduction of the mercury salt provided **54** (21 mg, 0.03 mmol, 52%) as a colorless oil. Data of inseparable mixture of isomers: R_f = 0.55 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3382, 2933, 2868, 1740, 1709 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.37−7.20 (m, 15H), 5.51−5.07 (m, 1H), 4.70−4.19 (m, 7H), 4.11−3.38 (m, 11H), 3.73 & 3.72 & 3.70 & 3.69 (s, 3H), 2.05−1.75 (m, 2H), 1.43 & 1.43 & 1.42 (s, 9H). HRMS (ESI) calculated for $C_{37}H_{48}NO_9$ $(M+H)^+$: 650.3329, found: 650.3320. HRMS (ESI) calculated for $C_{37}H_{47}NO_9Na$ $(M+Na)^+$: 672.3149, found: 672.3136.

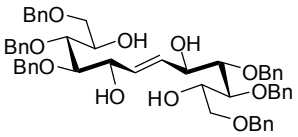
(3*S*,4*R*,5*R*)-3,4-Bis(benzyloxy)-5-(benzyloxymethyl)-2-ethyltetrahydrofuran-2-ol (30)



Compound **30** is one of the main byproducts that are formed during the cross metathesis reaction when using (2*R*,3*R*,4*R*,5*S*)-1,3,4-tris(benzyloxy)-6-

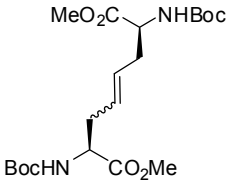
heptene-2,5-diol (**24**). R_f = 0.76 (EtOAc/heptanes, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.31−7.28 (m, 15H), 4.65−4.40 (m, 6H), 4.15−3.44 (m, 5H), 1.85−1.76 (m, 2H), 1.58 (s, 1H), 0.97 & 0.94 (2 x t, J = 5.4 Hz, 3H). Spectral data are in accordance with literature.[8]

(3*R*,4*R*,5*R*,8*R*,9*S*,10*S*,*E*)-1,3,4,9,10,12-Hexakis(benzyloxy)dodec-6-ene-2,5,8,11-tetraol (32)



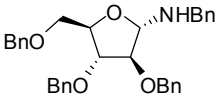
Compound **32** is one of the byproducts that are formed during the cross metathesis reaction when applying (2*R*,3*R*,4*R*,5S)-1,3,4-tris(benzyloxy)-6-heptene-2,5-diol (**24**). R_f = 0.30 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3430, 2920, 2863, 1455 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.33−7.20 (m, 30H), 5.90−5.88 (m, 2H), 4.69−4.57 (m, 7H), 4.51−4.36 (m, 7H), 4.11 (dt, *J* = 5.7, 3.5 Hz, 2H), 3.83 (ap t, *J* = 5.2 Hz, 2H), 3.75−3.45 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 139.8, 139.7, 133.5, 129.4, 129.3, 129.2, 129.0, 128.9, 128.8, 128.6, 83.2, 81.9, 75.2, 74.8, 74.3, 73.2, 72.8, 71.7. HRMS (ESI) calculated for $C_{54}H_{60}NO_{10}Na$ $(M+Na)^+$: 891.4084, found: 891.4001.

(2*R*,7*S*)-Dimethyl 2,7-bis(*tert*-butoxycarbonylamino)oct-4-enedioate (33)



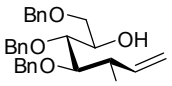
Compound **33** is one of the main byproducts that are formed during the cross metathesis reaction when using Boc-protected (*R*)-propargylglycine methyl ester (**25**). R_f = 0.62 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 3373, 2980, 1744, 1718 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.42−5.38 (m, 2H), 5.08 (d, *J* = 7.8 Hz, 2H), 4.31 (m, 2H), 3.73 (br s, 6H), 2.46−2.41 (m, 4H), 1.42 (s, 18H). HRMS (ESI) calculated for $C_{20}H_{35}N_2O_8$ $(M+H)^+$: 431.2393, found: 431.2374. HRMS (ESI) calculated for $C_{20}H_{35}N_2O_8Na$ $(M+Na)^+$: 453.2213, found: 453.2197.

2-Benzylamino-3,4,6-tri-*O*-benzylarabinose (55)



To a stirred suspension of tri-*O*-benzylarabinose (306 mg, 0.73 mmol) in MeOH (0.3 mL) was added benzylamine (115 mg, 1.1 mmol) at room temperature. Next, the mixture was heated to 55° C and stirred for 48 hours. Subsequently DCM (5 mL) was added and the resulting mixture was concentrated *in vacuo* to yield **55** as a yellow oil (369 mg, 0.72 mmol, 99%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.34−7.20 (m, 20H), 4.86 & 4.82 (d, *J* = 2.2 & 3.7 Hz, 1H), 4.70−4.46 (m, 7H), 4.28−3.80 (m, 5H), 3.66−3.50 (m, 2H). Spectral data are in accordance with literature.[15]

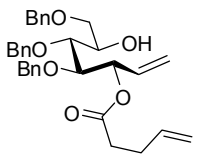
(2*R*,3*R*,4*R*,5*S*)-5-(Benzylamino)-1,3,4-tris(benzyloxy)-6-hepten-2-ol (57).



To a 1M solution of vinylmagnesium bromide (5.9 mL, 5.9 mmol) in dry THF (6 mL) was added a solution of $ZnBr_2$ (670 mg, 3.0 mmol) and the resulting mixture was stirred for 30 min to complete the divinylzinc formation. Next a solution of 2-benzylamino-3,4,6-tri-*O*-benzylarabinose (745 mg, 1.5 mmol) in THF (7 mL) was added and the reaction was stirred for 4 hours at room temperature. Subsequently the reaction was quenched by addition of a saturated NH_4Cl solution. The organic layer was diluted with DCM, washed with 5% HCl, saturated aqueous $NaHCO_3$ solution and water, dried ($MgSO_4$) and evaporated *in vacuo*. Purification *via* flash column chromatography afforded **57** (517 mg, 0.96 mmol, 66%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.32−7.19 (m, 20H), 5.78 (ddd, *J* = 17.1, 10.2, 8.7 Hz, 1H), 5.23 & 5.14 (dd, *J* = 10.3, 1.6 Hz & 17.2, 1.4 Hz, 2H),

4.69−4.38 (m, 6H), 4.01−3.54 (m, 7H), 3.48 & 3.34 (d, *J* = 2.2 Hz, 1H). Spectral data are in accordance with literature.[16]

(3*R*,4*R*,5*R*)-4,5,7-Tris(benzyloxy)-6-hydroxyhept-1-en-3-yl pent-4-enoate (61)



To a solution of 4-pentenoic acid (23 mg, 0.23 mmol) in DCM (2 mL) was added DCC (51 mg, 0.25 mmol) and DMAP (3 mg, 0.024 mmol) and the mixture was stirred for 20 min, next (2*R*,3*R*,4*R*,5*S*)-1,3,4-tris(benzyloxy)-6-heptene-2,5-diol (**24**) (100 mg, 0.22 mmol) was added and stirring continued for 2 hours. Evaporation of the solvent *in vacuo* and purification of the product by flash column chromatography afforded a slightly impure **61** (58 mg, 0.11 mmol, 48%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.37−7.25 (m, 15H), 5.92−5.73 (m, 2H), 5.68−5.64 (m, 1H), 5.34−5.16 (m, 2H), 5.09−4.95 (m, 2H), 4.71−4.50 (m, 6H), 4.03−3.98 (m, 1H), 3.85 (dd, *J* = 6.6, 3.7 Hz, 1H), 3.67 (dd, *J* = 6.9, 3.6 Hz, 1H), 3.64−3.58 (m, 2H), 2.61 (d, *J* = 5.7 Hz, 1H), 2.43−2.31 (m, 4H).

2.8 REFERENCES

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Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.

-Marie Curie

If I have a thousand ideas
and only one turns out to be good, I am satisfied.

-Alfred Nobel

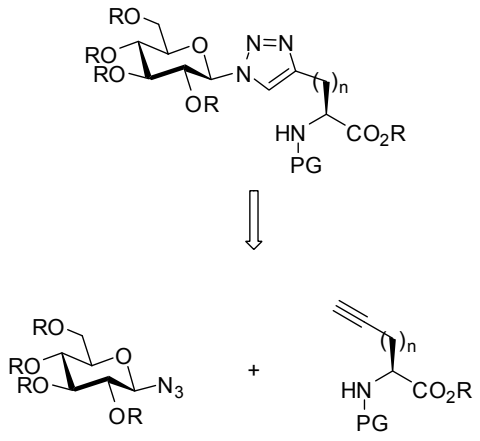
CHAPTER

3

Synthesis of Triazole-linked Glycoamino Acids

Abstract*:

An expedient, high-yielding synthesis of triazole-linked glycopeptides is described *via* Cu(I)–catalyzed [3+2] cycloaddition of azido–functionalized glycosides and acetylenic amino acids. Both α- and β-configured triazolyl glycopeptides were efficiently prepared by a variety of suitably functionalized (oligo)saccharides and (oligo)peptides, resulting in a novel class of stable glycopeptide mimics.



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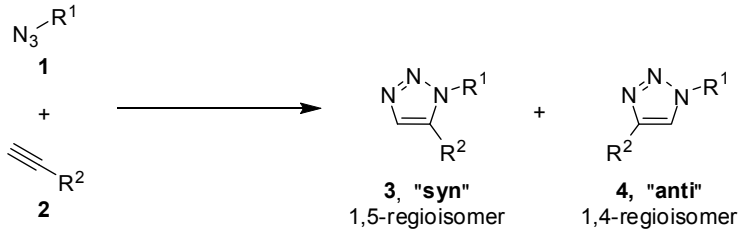
3.1 INTRODUCTION

1,3-Dipolar cycloaddition reactions have been applied for several years for the preparation of carbohydrate mimics.[1] Among the applications is the [3+2] Huisgen cycloaddition in the synthesis of *N*–glycosyl triazoles[2] as potential cytostatic agents. A drawback of the thermal Huisgen cycloaddition between an acetylene and an azide, however, is the inevitable mixture of two product regioisomers, the 1,4- and 1,5-disubstituted triazole compounds **4** and **3**, respectively (Scheme 1).[3] A valuable discovery was made in 2002, when Meldal[4] and Sharpless[5] independently described that a copper(I)-catalyzed procedure leads solely to the 1,4-disubstituted [1,2,3]-triazole **4**. Seldomly a novel reaction attained popularity so rapidly as the Cu(I)-catalyzed cycloaddition between an azide and an acetylene,[6,7] a reaction that is commonly referred to as 'click reaction' or more specifically CuAAC (copper-catalyzed azido acetylene [3+2] cycloaddition). Several factors are responsible for the strong interest in the CuAAC, such as the simple procedures for introduction of azides or alkynes into organic molecules, the practical simplicity and mildness of the condensation reactions, and the compatibility with water and/or a multitude of functional groups as present in biomolecules. In particular the latter reason has led to extensive application of the CuAAC for bioconjugation, for example as a result of the fact that both acetylenes and azides can be readily introduced into proteins, *e.g.* by protein engineering.[8]



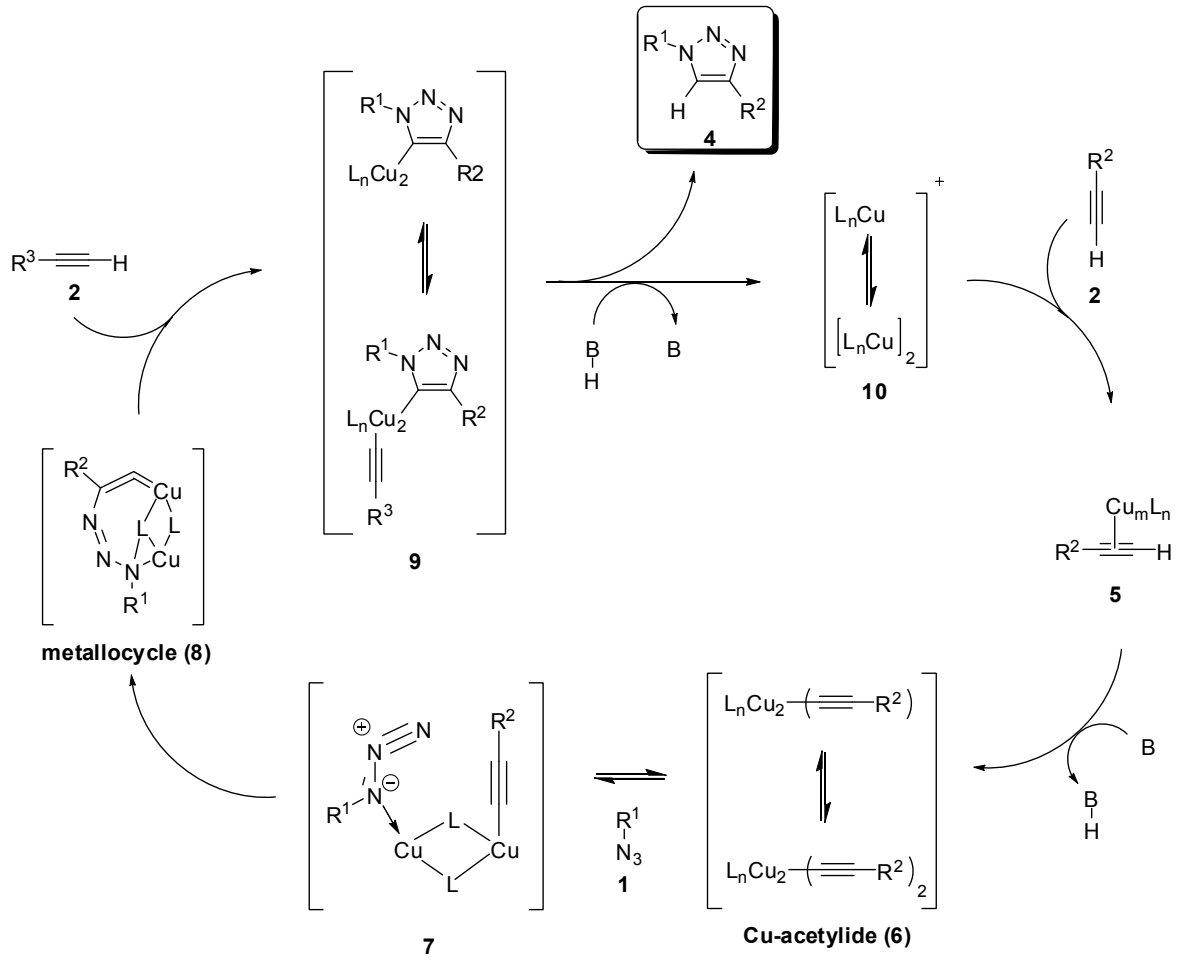
"Huisgen"	heat	*V*	:	*V*
"Sharpless / Meldal"	[Cu^{I}], rt	—	:	*V*

Scheme 1. *Thermal vs. Cu(I)-catalyzed cycloaddition.*

Although the 1,3-dipolar cycloaddition can be performed under the influence of various copper(I) sources (*e.g.* CuBr, CuI, CuCN, CuCl), best results are often achieved by *in vivo* generation of Cu(I) from a source of copper(II), *e.g.* $CuSO_4$ or $Cu(OAc)_2$, and a reducing agent (*e.g.* sodium ascorbate). The Cu(I)-catalyzed cycloaddition can be conducted in a variety of solvents (or mixtures thereof),

including water, alcohols, DMSO, DMF, MeCN and acetone. In most cases the reaction proceeds most quickly in an aqueous solvent mixture. However, due to the fact that Cu(I) is unstable in aqueous solvents, some of the more sluggish cycloadditions benefit from stabilizing ligands such as tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA).[9]

A proposed mechanism for the copper(I)–catalyzed azido acetylene cycloaddition, based on calculations and kinetic studies[6,10] is depicted in Scheme 2.



Scheme 2. *Proposed mechanism for the copper(I)–catalyzed azido acetylene cycloaddition.*

The mechanism starts with the formation of a Cu-acetylide species **6** *via* coordination of Cu(I) (**10**) with acetylene **2** leading to the π-complex **5**, followed by deprotonation and replacement of the acetylene proton. Next, an azide **1** replaces one of the ligands of the complex and binds to copper *via* the electron-rich nitrogen (**7**), thereby

inducing attack of the non-terminal acetylide carbon onto the remote nitrogen of azide, forming an unusual eight–membered metallocycle **8**. Metallocycle **8** has the correct conformation for ring–contraction leading to the copper–triazole species **9**. Proteolysis of **9** and thereby formation of the triazole product **4** completes the catalytic cycle and regenerates a Cu(I)-species **10** capable of starting a new cycle.

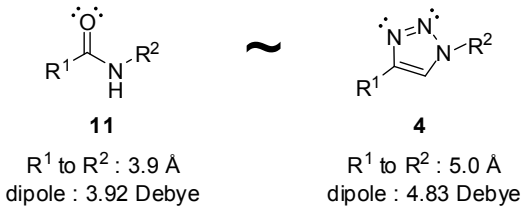
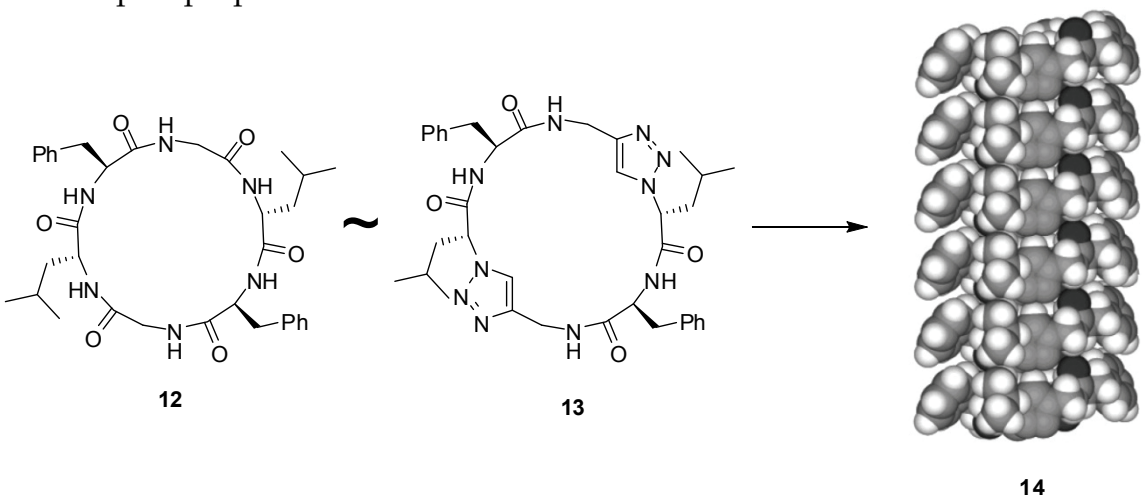


Figure 1. *Physical properties of a triazole compared to an amide.*

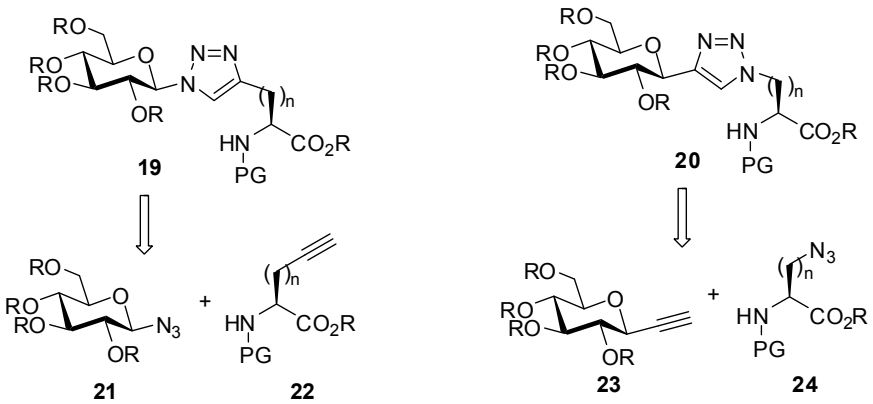
During the course of our investigations we became intrigued by the idea of a triazole function acting as an amide isostere,[6,11,12] in terms of electronic properties and placement of substituents, a hypothesis corroborated by studies of van Maarseveen *et al*.[12] Despite the common characteristics, some notable differences remain, in particular a calculated increase of distance of 1.1 Å between R^1 and R^2 for a triazole as compared to that of an amide bond (Figure 1). In addition, a triazole possesses a stronger dipole moment compared to an amide bond,[13] a feature that may actually enhance peptide bond mimicry by increasing the hydrogen bond donor and acceptor properties of a triazole.



Scheme 3. *Nanotube (**14**) formation from triazole containing octapeptide **13**.*

A seminal example showing the potential of triazoles acting as amide mimics was provided by the group of Ghadiri,[11] in a comparison of triazole-containing cyclic octapeptides **13** (Scheme 3) to natural peptides. It was found that triazole isosteres of peptides **13** behave highly similar to the natural non–triazole containing analogues **12**,[14] both forming an extended network resulting in solvent–filled nanotubes **14** (Scheme 3).

We hypothesized that triazole mimics of the natural amide linkage in *N*-glycoproteins would entail a novel class of stable glycoprotein analogues with potentially relevant biological properties. Modification of proteins by glycosylation is an essential post-translational process, and diverse and numerous examples have illustrated its key role in the modulation of protein properties.[15] For systematic study of the effects of protein glycosylation, efficient methods for the synthesis of pure, complex, differentially glycosylated proteins and mimics are therefore needed.[16] However, the synthesis of a typical glycoprotein is still far from trivial, while the final glycoproteins suffer from the inherent susceptibility to base-induced elimination (*O*-glycoprotein) or enzymatic hydrolysis (*N*-glycoprotein). Therefore, we became interested in an efficient, high-yielding synthesis of triazole-linked glycopeptides such as **19** and **20** (Scheme 4), as new and stable glycoamino acid isosteres, *via* the Cu–catalyzed procedure for the [3+2] cycloaddition between organic azides and acetylenes.



Scheme 4. *Retrosynthesis of glycoamino acids.*

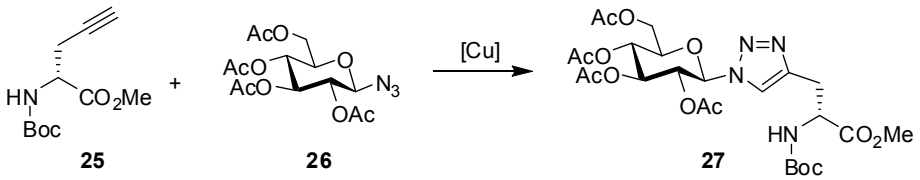
An additional stimulus to us was based on the fact that several of the building blocks required for the preparation of **19** and **20**, *i.e.* condensation of azidoglycosides **21** with acetylenic amino acids **22**, or acetylenic glycosides **23** with azide–containing

amino acids **24**, respectively, had already been prepared by us by (chemo)enzymatic means. Obviously, besides the aim to solely construct stable glycopeptide mimics, the resulting substituted triazoles may be applied for the investigation of glycoprotein function but may also serve as a novel compound class for pharmaceutical application, for example as vaccines[17] or for the treatment of AIDS.[18]

3.2 OPTIMIZATION OF REACTION CONDITIONS

As a model system to probe the viability of coupling azidosugars with organic aceylenes, azidoglucoside **26** and (*R*)-*N*-Boc-propargylglycine methyl ester (**25**) were chosen (Table 1). The cycloaddition was investigated by application of a variety of Cu(I) species (*e.g.*, CuI, CuCl, and CuCN) and bases (*e.g.* Et_3N and DIPEA). Entries 1 and 2 display the attempts to perform the cycloaddition without a base using a mixture of *tert*-butyl alcohol and water (entry 2) or the non-protic solvent THF (entry 1). As shown, only small amounts of the desired product could be isolated (2 to 10% yield).

Table 1. *Optimization of the reaction conditions.*



25 **26** **27**

entry	Cu-source	mol%	base / reductor	solvent	time	yield (%)
1	CuX[a]	20	–	THF	2 d	3-10
2	CuX[a]	20	–	^{t}BuOH:H_2O (1:1)	2 d	2-10
3	CuCN	20	20 equiv DIPEA	^{t}BuOH:H_2O (1:1)	3 d	50
4	CuI	20	0.4 equiv DIPEA	THF	1 d	64
5	CuI	20	20 equiv DIPEA	THF	5 h	63
6	Cu-wire	N.A.	20 equiv DIPEA	THF	2 d	~15
7	Cu-wire	N.A.	20 equiv DIPEA	THF	12 d	71
8	$Cu(OAc)_2$	20	Na ascorbate	^{t}BuOH:H_2O (1:1)	8 h	98
9	$Cu(OAc)_2$	1	Na ascorbate	^{t}BuOH:H_2O (1:1)	2 d	74

[a]X = Cl or I or CN

Clearly, addition of DIPEA as a base dramatically improved the outcome of the reaction (entry 3), giving the desired adduct **27** in a yield of 50% after stirring for 3

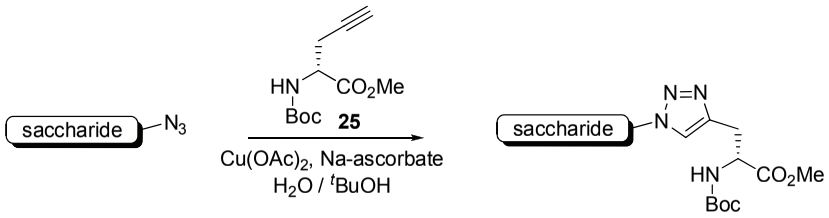
days. Similar results were obtained with CuI or copper wire in THF (entries 4-7). Additional equivalents of the base improved the reaction rate, but had negligible influence on the yield of the reaction. Optimal results were obtained by addition of 0.2 equiv of $Cu(OAc)_2$ and 0.4 equiv of sodium ascorbate in a 1:1 *(v/v)* mixture of water and *tert*-butyl alchohol (entry 8), providing the targeted glycoamino acid **27** in 98% yield. Reduction of catalyst loading (entry 9) resulted in prolonged reaction time and reduced yield.

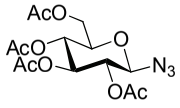
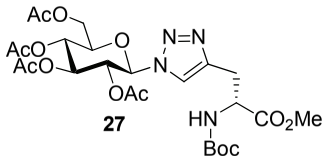
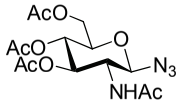
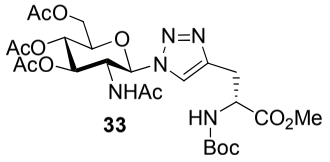
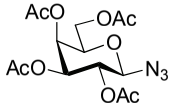
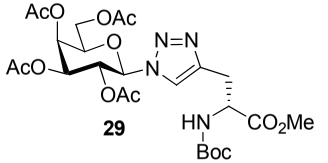
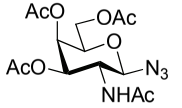
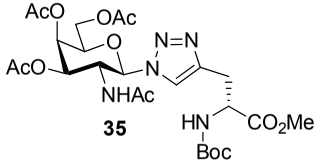
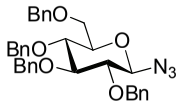
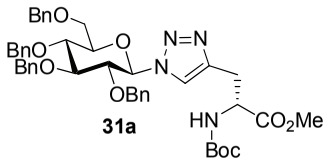
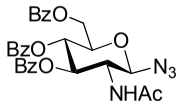
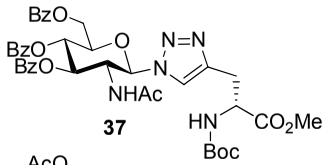
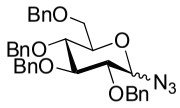
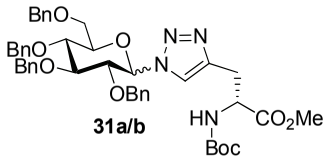
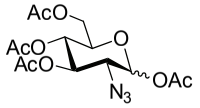
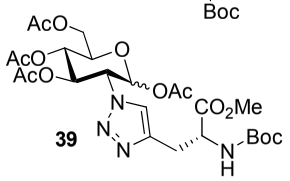
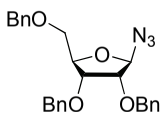
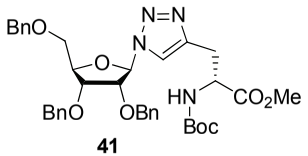
3.3 SYNTHESIS OF TRIAZOLE-LINKED GLYCOPEPTIDES

3.3.1 CuAAC of various sugar azides

Having optimized the [3+2] cycloaddition for azidoglucoside **26** and (*R*)-*N*-Boc-propargylglycine methyl ester (**25**), the scope of the CuAAC reaction for the preparation of a variety of glycoamino acids was explored. To this end, several different protected azidosugars (Table 2) were prepared *via* literature procedures and subjected to [3+2] cycloaddition with (*R*)-*N*-Boc-propargylglycine methyl ester (**25**) under the optimal conditions (0.2 equiv of $Cu(OAc)_2$ and 0.4 equiv of sodium ascorbate in a 1:1 (*v/v*) mixture of water and *tert*-butyl alchohol). First of all, condensation of *galacto*-configured azide **28** (entry 2) proceeded similarly smooth as the model *gluco*-configured model glycoside **26** (entry 1) to provide the targeted glycoamino acid **29** in 89% yield. Under the same conditions, the more sterically hindered and more electron-rich benzylated glycosylazide **30a** also led to the corresponding adduct **31a** in excellent yield. As shown, the same azide as a mixture of anomers **30a/b** (α:β = 1:4) gave the triazolyl product **31a/b** in the same anomeric ratio and yield as the starting azides (entry 4), providing a strong indication that α and β-anomers react similarly. Since the first carbohydrates on *N*- and *O*-glycoproteins in Nature are mostly formed by *N*-acetyl-β-D-glucosamine or *N*-acetyl-α-D-galactosamine, we were intrigued whether 1-azido-*N*-acetylglucosamine (**32**) and 1-azido-*N*-acetylgalactosamine (**34**) would also be compatible with the conditions of the [3+2] cycloaddition.

Table 2. *Variation of the saccharide moiety.*



entry	azido saccharide	glycopeptide[a,b]	yield	entry	azido saccharide	glycopeptide[a,b]	yield
1	 **26**	 **27**	(98%)	5	 **32**	 **33**	(91%)
2	 **28**	 **29**	(89%)	6	 **34**	 **35**	(72%)
3	 **30a**	 **31a**	(88%)	7	 **36**	 **37**	(30%)
4	 **30a/b** (α:β 1:4)	 **31a/b**	(87%) (α:β 1:4)	8	 **38** (α:β 1:6)	 **39**	(71%) (α:β 1:6)
				9	 **40**	 **41**	(88%)

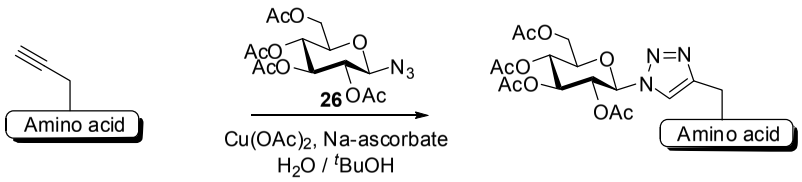
[a]Reagents and conditions: 1 equiv azidoglycoside, 1 equiv amino acid derivative **25** , 0.2 equiv $Cu(OAc)_2$, 0.4 equiv Na-ascorbate, $H_2O/^tBuOH$, 1:1 (v/v), rt, 16 h. [b]Yield of isolated product.

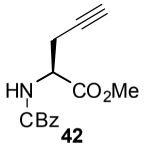
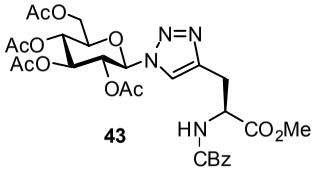
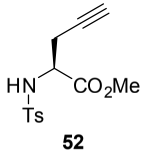
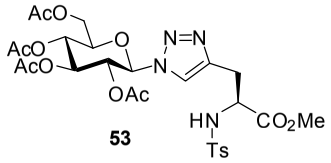
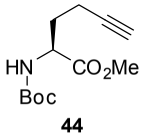
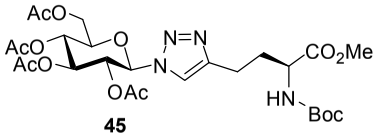
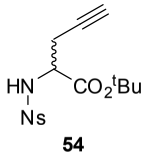
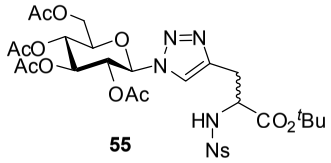
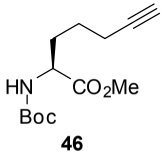
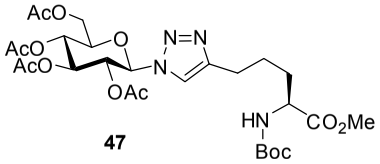
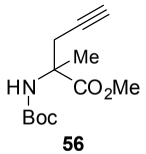
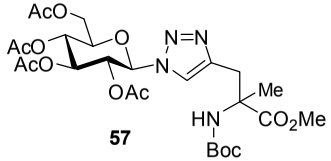
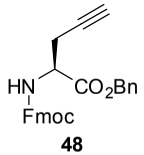
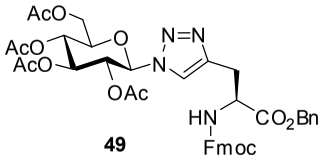
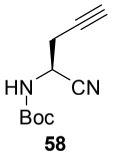
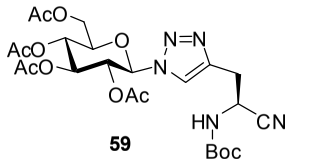
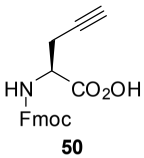
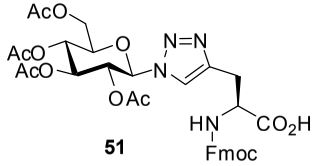
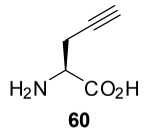
Therefore, the preparation of **32** and **34** was first undertaken by subjecting *N*-acetylglucosamine and *N*-acetylgalactosamine to Shiozaki conditions[19] (with the slight modification of substituting pure tin(IV)chloride by a 1 M solution in DCM), but complex mixtures of inseparable compounds were obtained. The desired 1-azidosugars **32** and **34** could be prepared, however, by S_N2 displacement of the corresponding anomeric chlorides, in turn readily obtained by treatment of the *N*-acetylated aminosugar with neat acetyl chloride. Gratifyingly, these azides reacted efficiently with the acetylenic amino acid **25** to give the corresponding products **33** and **35** in good yields (entries 5 and 6). Reaction of the benzoyl-protected glucosamine **36** proceeded smoothly as well, leading to rapid conversion of starting material, although partial decomposition during work-up and column chromatography resulted in a somewhat lower yield of glycoamino acid **37** (entry 7). The glucose derivative **38** containing a 2-azido function shows the possibility of introducing the triazole to other positions of the carbohydrate (as reported by Meldal),[4] giving the desired coupling product **39** in 71% yield (entry 8). Finally, condensation of 5-ring azidofuranose **40** leading to the triazolyl glycoamino acid **41** (entry 9) proceeded uneventfully. Clearly, there were no noticeable differences in reactivity between the several monosaccharides or between the α- and β-isomers.

3.3.2 CuAAC of various amino acids

Besides variation of the saccharide moiety, the compatibility of a diversity of amino acid protective groups with the established conditions was also investigated. Several of the non-proteinogenic amino acids were available to us, while others were readily prepared by known methods.[20] A variety of *N*-protective groups was investigated, including Fmoc (**48** and **50**), tosyl (**52**), nosyl (**54**), and Cbz (**42**). Additionally, various carboxyl protecting groups were applied including a benzyl (**48**) and a *tert*-butyl (**54**) ester. Substrates of various chain-length were included (**44** and **46**), as well as α-methyl propargylglycine (**56**) and an acetylenic aminonitrile (**58**). The results of condensing the array of various acetylenic amino acids under the optimal conditions (0.2 equiv of $Cu(OAc)_2$ and 0.4 equiv of sodium ascorbate in a 1:1 (*v/v*) mixture of water and *tert*-butyl alcohol) with azidoglucoside **26** are displayed in Table 3. Entries 1-2 and 5-7 show that besides the Boc also Cbz, Fmoc, Ts and Ns are well-suited, providing the desired products in moderate to good yields (50–91%).

Table 3. *Variation of the amino acid moiety.*



entry	amino acid acetylene	glycopeptide[a,b]	yield	entry	amino acid acetylene	glycopeptide[a,b]	yield
1	 **42**	 **43**	(50%)	6	 **52**	 **53**	(64%)
2	 **44**	 **45**	(80%)	7	 **54**	 **55**	(87%)
3	 **46**	 **47**	(76%)	8	 **56**	 **57**	(85%) (R:S 1:1)
4	 **48**	 **49**	(70%)	9	 **58**	 **59**	(60%)
5	 **50**	 **51**	(91%)	10	 **60**		**No product**

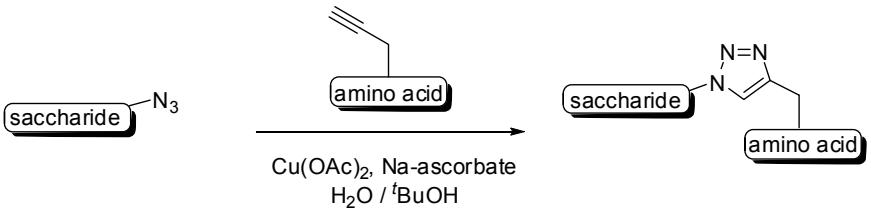
[a]Reagents and conditions: 1 equiv. azidoglycoside **26**, 1 equiv. amino acid, 0.2 equiv. $Cu(OAc)_2$, 0.4 equiv. Na-ascorbate, $H_2O/^tBuOH$ 1:1 (v/v), rt, 16 h.
[b]Yield of isolated product.

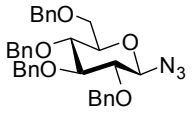
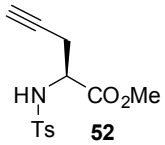
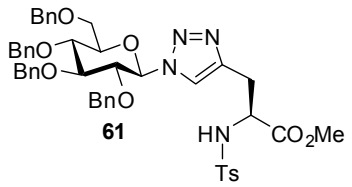
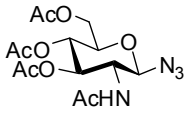
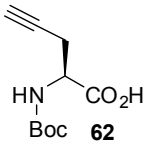
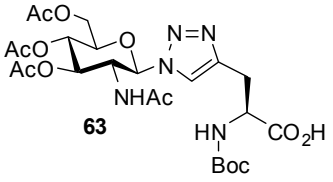
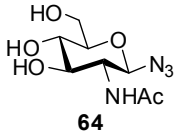
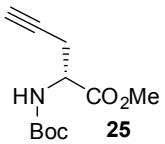
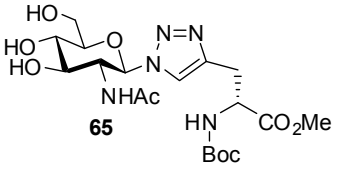
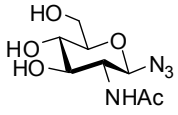
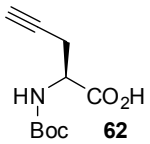
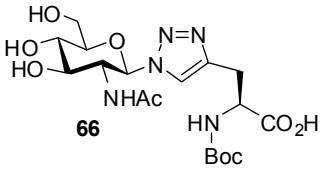
The somewhat lower yield of the Cbz protected product **43** as compared to the Boc-protected analogue was probably due to solubility problems of the starting material. In addition to the methyl ester, a variety of different carboxyl protecting groups appeared perfectly stable under the reaction conditions, such as benzyl (**49**) and *tert*-butyl esters (**55**). Increasing the length of the side chain, as for (*R*)-*N*-Boc-homopropargylglycine methyl ester **44** or (*R*)-*N*-Boc-bishomopropargylglycine methyl ester **46** (entries 2 and 3) showed no significant change in reactivity, providing products **45** and **47** in good yields. Similarly, α-methyl–substituted propargylglycine **56** (entry 8) afforded the glycoamino acid **57** in 85% yield, but application of the acetylenic aminonitrile **58** afforded the desired adduct **59** in somewhat lower yield (60%). Summarizing, a wide range of carboxyl and *N*-protective groups is suitable for CuAAC with azidosugars, but amino acids lacking an amino protective group **60** could not be successfully coupled.

3.3.3 Other combinations of azidosugars and acetylenic amino acids

Besides the triazolyl glycoamino acids described above, a few additional combinations of azidosugars and acetylenic amino acids were subjected to copper(I)-catalyzed cycloaddition (Table 4). Not surprisingly, cycloaddition of previously applied substrates (entries 1 and 2) proceeded uneventfully. To validate the use of unprotected carbohydrates azidoglucosamine **32** was hydrolyzed using a solution of K_2CO_3 in MeOH affording the *O*-unprotected azidosugar **64**. Remarkably, Cu(I)-catalyzed cycloaddition with unprotected sugar **64** and (*R*)-*N*-Boc-propargylglycine methyl ester **25** (entry 3) also proceeded smoothly, leading to quantitative formation of the desired compound **65** as indicated by TLC analysis. However, due to the high water solubility of compound **65**, it could not be isolated in sufficiently purified form. Finally, the unprotected sugar **64** was also reacted with propargylglycine **62** containing a free carboxylic acid, again giving full conversion to the desired product **66** as shown by TLC.

Table 4. *Other triazolyl glycoamino acids via CuAAC.*



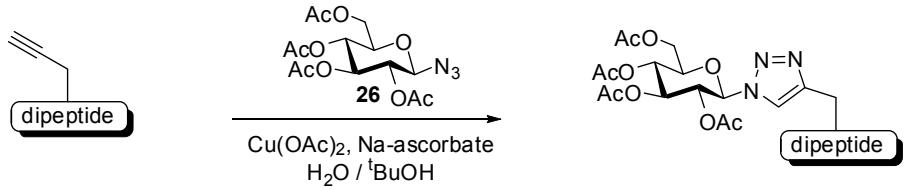
entry	azido saccharide	amino acid acetylene	glycopeptide[a]	yield[b]
1	 **30a**	 **52**	 **61**	(82%)
2	 **32**	 **62**	 **63**	(86%)
3	 **64**	 **25**	 **65**	(n.i.)
3	 **64**	 **62**	 **66**	(53%)

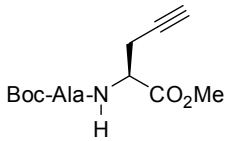
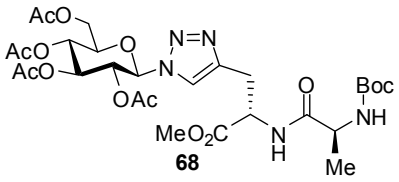
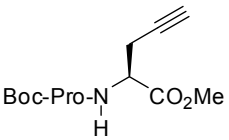
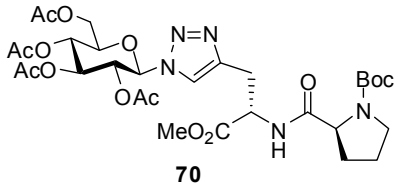
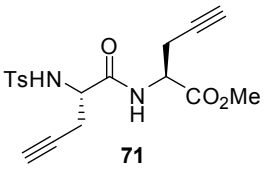
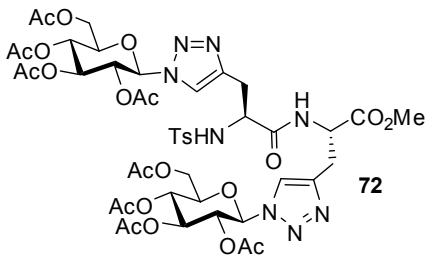
[a]Reagents and conditions: 1 equiv azidoglycoside **30a**, **32** or **64**, 1 equiv amino acid **25**, **52** or **62**, 0.2 equiv $Cu(OAc)_2$, 0.4 equiv Na-ascorbate, H_2O/^{t}BuOH, 1:1 (*v/v*), rt, 16 h. [b]Yield of isolated product.

Purification was now effected with ion-exchange chromatography, albeit that only a moderate yield (53%) of **66** was obtained. An easier and higher yielding route towards unprotected triazolyl glycoamino acids is based on cycloaddition of the acetyl-protected analogues, followed by deprotection with K_2CO_3 in MeOH.

3.3.4 CuAAC of dipeptides and disaccharides

Table 5. *Cycloaddition reactions of dipeptides.*

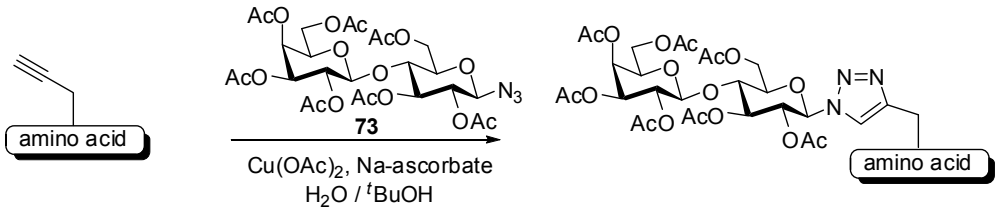


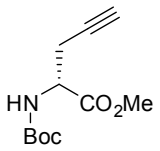
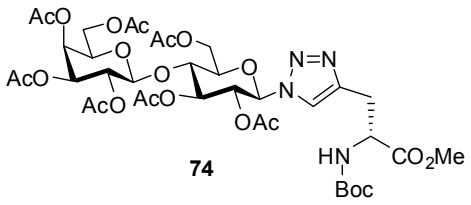
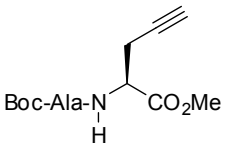
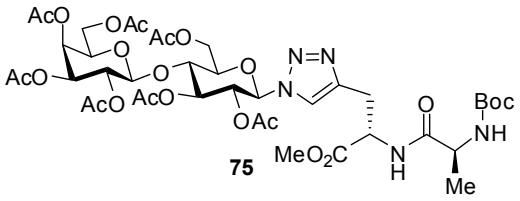
entry	acetylenic dipeptide	glycopeptide[a]	yield[b]
1	 **67**	 **68**	(86%)
2	 **69**	 **70**	(92%)
3	 **71**	 **72**	(55%)[c]

[a]Reagents and conditions: 1 equiv azidoglycoside **26**, 1 equiv dipeptide, 0.2 equiv $Cu(OAc)_2$, 0.4 equiv sodium ascorbate, H_2O/^{t}BuOH 1:1 (*v/v*), rt, 16 h. [b]Yield of isolated product. [c]2 equiv azidoglycoside **26** was used.

As a first study towards the assembly of complex triazolyl glycopeptides, it is essential that CuAAC is not limited to single amino acids or saccharides, but can be efficiently extended to oligopeptides and olgiosaccharides, respectively. Thus, dipeptides **67, 69** and **71** were prepared *via* standard coupling procedures, and served as simple model peptides to explore the suitability of CuAAC for the preparation of glycoproteins (Table 5). For example, the two protected dipeptides **67** and **69** were successfully coupled to glycosyl azide **26**, affording the glycopeptides **68** and **70** in 86 and 92% yield, respectively.

Table 6. *Cycloaddition reactions involving a disaccharide.*



entry	amino acid acetylene	glycopeptide[a]	yield[b]
1	 **25**	 **74**	(83%)
2	 **67**	 **75**	(81%)

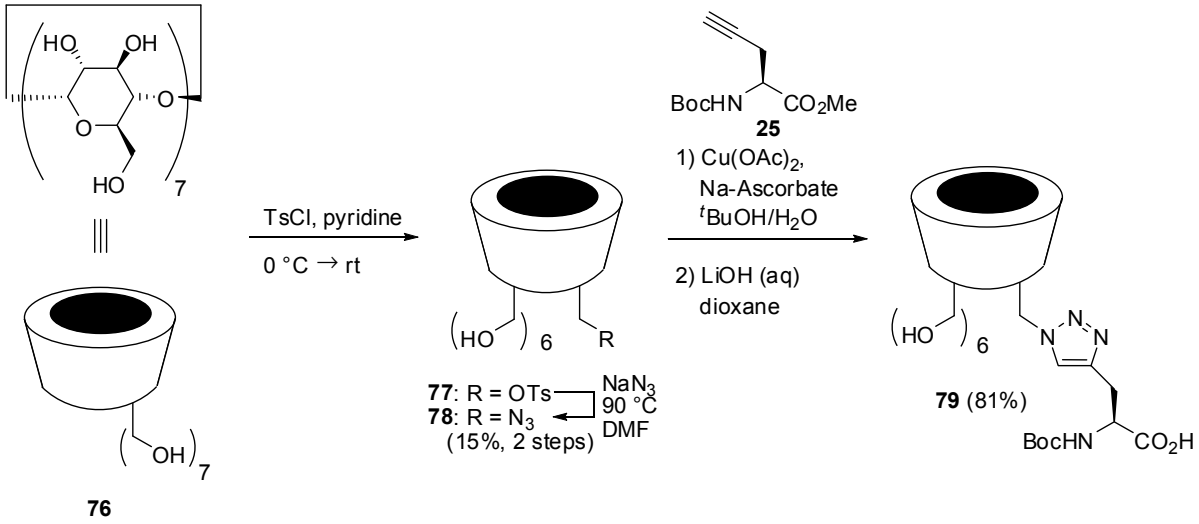
[a]Reagents and conditions: 1 equiv azidoglycoside **73**, 1 equiv amino acid, 0.2 equiv $Cu(OAc)_2$, 0.4 equiv Na-ascorbate, H_2O/^{t}BuOH 1:1 (*v/v*), rt, 16 h. [b]Yield of isolated product.

The dipeptide **71** bearing two adjacent acetylene functionalities could also be readily reacted with azidosugar **26.** The diglycosylated dipeptide **72** was successfully obtained, although the reaction proceeded considerably slower and therefore led to the desired product in somewhat lower yield (55%), possibly due to increased steric interactions. Inversely, the disaccharide azide **73** was coupled to amino acid **25** or dipeptide **67** to give the corresponding triazoles **74** and **75** with satisfactory yields (Table 6).

3.3.5 CuAAC involving β-cyclodextrin and an oligopeptide

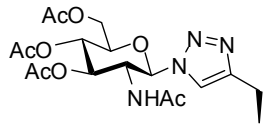
Considering the fact that disaccharides and dipeptides coupled smoothly under the action of Cu(I)-catalysis, the stage was set to investigate the synthesis of triazole compounds involving larger and more complex structures like oligosaccharides and oligopeptides. To this end, mono-6-*O*-azido-β-cyclodextrin was chosen as a readily accessible model oligosaccharide. Treatment of dried β-cyclodextrin **76** with tosyl choride (0.9 equiv) in pyridine provided mono-6-*O*-tosylated β-cyclodextrin **77** as the

main product (Scheme 5), in combination with amounts of di- and trisubstituted products. Because separation of the differentially substituted products failed to proceed satisfactorily, the crude mixture containing **77** was treated with NaN_3 in DMF to provide the mono- , di- and trisubstituted azides. The desired monoazido-β-cyclodextrin **78** could now be separated from the other azides by repetitive flash column chromatography, providing compound **78** in 15% over the two steps.



Scheme 5. *Cu(I) catalyzed cycloaddition of mono azido-β-cyclodextrin.*

Compound **78** is a completely unprotected heptasaccharide and therefore suitable to serve as a typical oligosaccharide prototype. In order to establish whether CuAAC of **78** would occur with an azidofunctionalized amino acid, Boc-protected propargylglycine methyl ester (**25**) was stirred with monoazido-β-cyclodextrin **78** under the standard conditions with $Cu(OAc)_2$ and sodium ascorbate in a mixture of *tert*-butanol and water (1:1), leading to a novel compound as judged by TLC analysis. Subsequent hydrolysis of the methyl ester and purification by ion exchange column chromatography provided a white fluffy substance, NMR analysis of which showed the typical triazole singlet at 7.64 ppm (^{1}H NMR). Moreover, high resolution mass spectrometry revealed that a compound had been formed with the accurate mass of the expected product **79**, isolated in a yield of 81%. It is therefore safe to conclude that copper(I)-catalyzed azide acetylene cycloaddition can be perfectly applied to oligosaccharide systems.



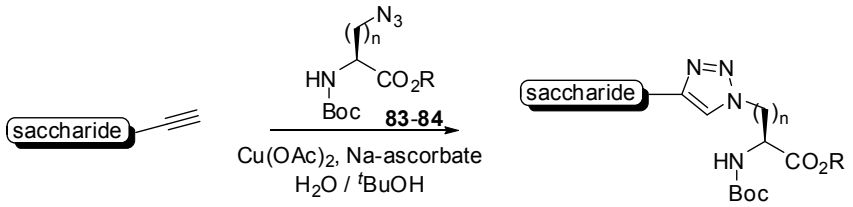
Ac-W-M-E-W-D-R-E-I-N-N-Y-T-S-L-I-H-S-L-I-E-E-S-Q-N-Q-Q-E-K-N-E-Q-E-L-L-NH_2

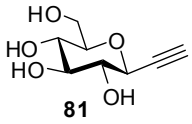
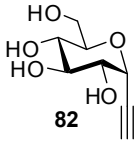
Figure 2. *Glycosylated peptide analogue of HIV fragment C34 (**80**).*

The final question to be answered is whether CuAAC is also suitable for synthesis of longer chain oligopeptides. Thus, a 34-mer fragment of the HIV gp41 glycoprotein[21] was synthesized on solid phase. Cycloaddition to azidoglucosamine **32** under the typical conditions proceeded successfully leading to the desired 34-mer glycosylated peptide **80**[22] (Figure 2).

3.3.6 *C*–linked triazolyl glycoamino acids

Table 7. *Synthesis of triazole-linked glycosidic amino acids from acetylene containing saccharides.*[23]



entry	acetylenic saccharide	amino acid	n	R	glycopeptide[a]	yield[b]
1		**83a**	1	H	**85a**	70%
2		**83b**	2	H	**85b**	73%
3	**81**	**83c**	3	H	**85c**	71%
4		**84**	1	Me	**86**	84%
5		**83a**	1	H	**87a**	60%
6	**82**	**83b**	2	H	**87b**	60%

[a]Reagents and conditions: 1 equiv acetylenic glycoside, 1 equiv amino acid, 0.2 equiv $Cu(OAc)_2$, 0.4 equiv Na ascorbate, H_2O/^{t}BuOH 1:1 (*v/v*), rt, 16 h. [b]Yield of isolated product.

Since all examples so far comprise the combination of azidoglycosides with acetylenic amino acids, we were intrigued whether ligation of constituents with inverted functionality, with the triazole N^1 position bound to the amino acid, would

also be feasible. Therefore, a set of acetylenic glycosides and azide–containing amino acids were prepared and it was shown that *N*-linked triazolyl glycopeptides can also be readily obtained *via* the Cu-catalyzed [3+2] cycloaddition. Both α- and β-glycopeptide products (**85–87**) were formed uneventfully in yields ranging from 60 to 84% (Table 7). In general, the slighly lower yields for the amino acid derivatives containing a free carboxylic acid can be rationalized by the somewhat more cumbersome purification as compared to the corresponding methyl ester counterparts. Furthermore, virtually no change in reactivity was observed upon elongation of the amino acid side chain as can be inferred from Table 7, entries 1-3.

3.4 CONCLUSION

In conclusion, we have developed a straightforward, versatile and high yielding method for the synthesis of a novel class of glycopeptides, involving the copper(I)-catalyzed azide-alkyne cycloaddition. The resulting triazole-linked products, mimicking the *N*-linked glycosides found in Nature, have potential biological properties. By application of this method a small library of glycosidic amino acids was prepared by combination of a variety of azido-functionalized glycosides with a range of acetylenic amino acids. We have shown that a wide range of saccharide as well as amino acids protecting groups are compatible with the conditions used. Furthermore we demonstrated that neither the chain-length of the amino acids nor the anomeric configuration of the saccharide moiety significantly influences reaction rates. Besides single amino acids and saccharides, more complex (oligo)peptides and (oligo)saccharides can be successfully ligated in this manner.

3.5 ACKNOWLEDGMENT

A. (Bram) R. Keereweer is gratefully acknowledged for his large contribution to this chapter. S. (Bas) A. M. W. van den Broek, Bart C. J. van Esseveldt and Bart Verheijen are kindly acknowledged for the preparation of several of the starting compounds. S. Groothuys, W. Adriaens and Dr. T. M. Hackeng are kindly thanked for their contribution to the synthesis of the C34 glycopeptide **80**. S. Groothuys is acknowledged for his work on the *C*-linked triazolyl glycoamino acids described in the section 3.3.6.

3.6 EXPERIMENTAL

General information. For general experimental details, see Section 2.6

Nomenclature of the triazole-linked glycosidic amino acids. For the sake of clarity the names of the triazole-linked glycosidic amino acids/peptides in the experimental part of this Thesis were abbreviated, applying the standard three letter codes for natural amino acids. For the non-proteinogenic amino acids we derived a code as depicted in Figure 6. Propargylglycine was abbreviated as Pgl, T4M stands for a triazol-4-yl-methylglycine-containing amino acid (Figure 3) and triazol-1-yl-butylglycine was for example abbreviated as T1B. Any substituents are presented in brackets.

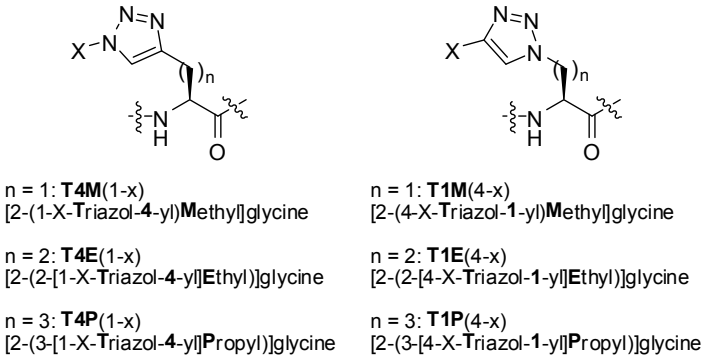
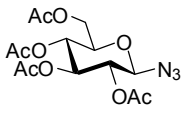


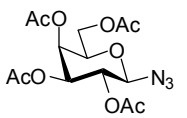
Figure 3. *Nomenclature of the triazole-linked glycosidic amino acids.*

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl azide (26)



Trimethylsilyl azide (238 µL, 1.79 mmol) and tin tetrachloride (930 µL, 1 M in DCM) were added at room temperature to a solution of 1,2,3,4,6-penta-*O*-acetyl-D-glucopyranose (500 mg, 1.28 mmol) in DCM (4 mL). Additional DCM was added after stirring the reaction mixture for 45 min and the solution was washed with H_2O, aqueous 1 M HCl and saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford **26** (472 mg, 1.26 mmol, 99%) as a white solid. R_f = 0.52 (EtOAc/heptane, 1/1). FTIR (ATR): ν = 2111, 1748, 1364, 1238, 1208 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 5.21 (app t, *J* = 9.5 Hz, 1H), 5.01 (app t, *J* = 9.7 Hz, 1H), 4.95 (dd, *J* = 9.5, 8.9 Hz, 1H), 4.64 (d, *J* = 8.9 Hz, 1H), 4.27 (dd, *J* = 12.5, 4.8 Hz, 2H), 4.17 (dd, *J* = 12.5, 2.3 Hz, 1H), 3.79 (ddd, *J* = 10.0, 4.8, 2.3 Hz, 1H), 2.10 (s, 3H), 2.07 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H). Spectral data are in accordance with literature.[24]

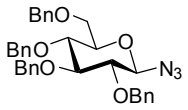
2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl azide (28)



Trimethylsilyl azide (0.8 mL, 6.08 mmol) and tin tetrachloride (3.8 mL, 1M in DCM) were added at room temperature to a solution of 1,2,3,4,6-penta-*O*-acetyl-D-galactopyranose (2.02 g, 5.17 mmol) in DCM (20 mL). Additional DCM was added after stirring the reaction mixture for 45 min and the solution was washed with H_2O, aqueous 1 M HCl and saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford **28** (1.92 g, 5.15

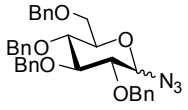
mmol, 99%) as a white solid. R_f = 0.42 (EtOAc/heptane, 1/1). FTIR (ATR): ν = 2120, 1735, 1372, 1216 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 5.43 (dd, *J* = 3.2, 1.2 Hz, 1H), 5.17 (dd, *J* = 10.4, 8.8 Hz, 1H), 5.04 (dd, *J* = 10.4, 3.6 Hz, 1H), 4.60 (d, *J* = 8.4 Hz, 1H), 4.17 (dd, *J* = 7.2, 3.2 Hz, 2H), 4.02 (dt, *J* = 6.6, 1.2 Hz, 1H), 2.17 (s, 3H), 2.10 (s, 3H), 2.07 (s, 3H), 1.99 (s, 3H). Spectral data are in accordance with literature.[25]

2,3,4,6-Tetra-*O*-benzyl-β-D-glucopyranosyl azide (30a)



A solution of 2,3,4,6-tetra-*O*-benzyl-D-glucose (222.6 mg, 0.41 mmol) in DCM (10 mL) and DMF (0.40 ml, 5.15 mmol), was treated with oxalyl chloride (0.5 mL, 5.26 mmol) and stirred for 4 h. Concentration of the reaction mixture *in vacuo* afforded the crude sugar halide. Next, the sugar halide was treated with NaN_3 (30 mg, 0.46 mmol) in DMF (5 mL) and stirred for 4 h at room temperature before concentrating the organic layer to a small volume *in vacuo*. EtOAc was added and the mixture was washed with H_2O, aqueous 1 M HCl, and saturated aqueous $NaHCO_3$, dried (Na_2SO_4) and concentrated *in vacuo* to afford **30a** (207 mg, 0.37 mmol, 90%) as a white solid. R_f = 0.82 (EtOAc/heptane, 1/1). FTIR (ATR): ν = 3347, 2907, 2111 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.35−7.22 (m, 18H), 7.15−7.08 (m, 2H), 4.90−4.71 (m, 5H), 4.63−4.50 (m, 4H), 3.72−3.59 (m, 2H), 3.49−4.39 (m, 1H), 3.38−3.33 (m, 1H). Spectral data are in accordance with literature.[26]

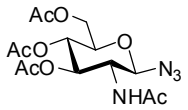
2,3,4,6-tetra-*O*-benzyl-α,β-D-glucopyranosyl azide (30a/b)



(α:β 1:4)

To a solution of 2,3,4,6-tetra-*O*-benzyl-D-glucose (179 mg, 0.33 mmol) in DMF (5 mL) was added DBU (82 µL, 0.60 mmol) and DPPA (168 mg, 0.61 mmol) and the mixture was stirred for 12 h. Subsequently, EtOAc was added and the mixture was washed with H_2O, aqueous 1 M HCl, and saturated aqueous $NaHCO_3$, dried (Na_2SO_4) and concentrated *in vacuo.* The crude product was purified by flash column chromatography (EtOAc/heptane, 1/3)to afford **30a/b** (150 mg, 80%) as a mixture of isomers (α:β 1:4). For spectral data of the major isomer see **30a**.

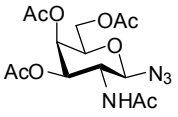
2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl azide (32)



1,2,3,4,6-Penta-*O*-acetyl-D-glucopyranose (500 mg, 1.28 mmol) was dissolved in acetyl chloride (4 mL) and stirred until it became a thick pink mass. Next, again 4 mL of acetyl chloride was added and the mixture was stirred overnight and subsequently diluted with ice–cold DCM, and poured into an $NaHCO_3$ ice–bath. The crude α–chloride was extracted with EtOAc, washed with ice–cold brine, dried (Na_2SO_4) and concentrated *in vacuo*. Then it was redissolved in DMF (5 mL), NaN_3 (125 mg, 1.93 mmol) was added and the mixture was stirred overnight at room temperature The mixture was concentrated *in vacuo* and taken up in EtOAc and H_2O. The aqueous layer was extracted with EtOAc and the combined organic layers were washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4) and concentrated *in vacuo.* The crude product was purified by flash column chromatography (EtOAc/heptane, 2/1) to obtain **32** (73.1 mg, 0.19 mmol, 15%) as a white solid. R_f = 0.10 (EtOAc/heptane, 1/1). FTIR (ATR): ν = 2115, 2098, 1744, 1221 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 5.66 (d, *J* = 8.9 Hz, 1H), 5.24 (dd, *J* = 10.6, 9.3 Hz, 1H), 5.10 (dd, *J* = 10.0, 9.4 Hz, 1H), 4.76 (d, *J* = 9.3 Hz, 1H), 4.28 (dd, *J* = 12.5, 4.8 Hz, 1H), 4.17 (dd, *J* = 12.4, 2.4 Hz, 1H), 3.94−3.87 (m, 1H), 3.79(ddd, *J* = 10.4, 4.8, 2.4 Hz,

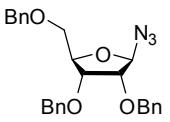
1H), 2.10 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.0, 170.7, 170.5, 169.3, 88.4, 74.0, 72.2, 68.1, 61.9, 54.2, 23.2, 20.7, 20.6, 20.5. Spectral data are in accordance with literature.[24]

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-galactopyranosyl azide (34)



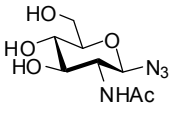
Galactosamine (249 mg, 1.12 mmol) was dissolved in acetyl chloride (4 mL) and stirred until the mixture became a thick pink mass. Next another 4 mL of acetyl chloride was added, the mixture was stirred overnight and subsequently diluted with ice–cold DCM, and poured into an $NaHCO_3$ ice–bath. The crude α–chloride was extracted with EtOAc, washed with ice–cold brine, dried (Na_2SO_4) and concentrated *in vacuo*, and redissolved in DMF (5 mL). NaN_3 (125 mg, 1.93 mmol) was added and the mixture was stirred overnight at room temperature The mixture was concentrated *in vacuo* and taken up in EtOAc and H_2O. The aqueous layer was extracted with EtOAc and the combined organic layers were washed with saturated aqueous $NaHCO_3$, brine, dried (Na_2SO_4) and concentrated *in vacuo.* The crude product was purified by flash column chromatography (EtOAc/heptane, 2/1) to obtain **34** (20 mg, 0.05 mmol, 5%) as a white solid. R_f = 0.13 (EtOAc/heptane, 1/1). FTIR (ATR): ν = 2111, 1739, 1364, 1225 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.58 (d, *J* = 8.8 Hz, 1H), 5.38 (dd, *J* = 3.3, 0.8 Hz, 1H), 5.24 (dd, *J* = 11.2, 3.3 Hz, 1H), 4.79 (d, *J* = 9.2 Hz, 1H), 4.17−4.15 (m, 2H), 4.03−3.99 (m, 2H), 2.16 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H). Spectral data are in accordance with literature.[27]

2,3,5-Tri-*O*-benzyl-β-D- ribofuranosyl azide (40)



Acetic anhydride (2 mL) was added to a solution of 2,3,5-tri-*O*-benzyl ribose (102 mg, 0.24 mmol) in pyridine (1 mL), the mixture was stirred overnight at room temperature. Saturated aqueous $NaHCO_3$ was carefully added and 1–*O*–acetyl–2,3,5–tri–*O*–benzyl ribose was extracted with DCM (3 ×). After purification with flash column chromatography (94 mg, 0.20 mmol, 84%), the compound was dissolved in dry DCM (1 mL) and , trimethylsilyl azide (35 µL, 0.26 mmol) and tin tetrachloride (20 µL, 1M in DCM) were added at room temperature. Next, the reaction mixture was stirred for 45 min, DCM was added and the solution was washed with H_2O, aqueous 1M HCl and saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4), filtered and concentrated *in vacuo.* The crude product was purified by flash column chromatography to afford **40** (85 mg, 0.19 mmol, 79% 2 steps) as a white solid. R_f = 0.26 (EtOAc/heptane, 1/3). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.38−7.28 (m, 15H), 5.35 (d, *J* = 2.1 Hz, 1H), 4.36 (ddd, *J* = 6.8, 4.6, 3.3 Hz, 1H), 4.67−4.47 (m, 6H), 4.08 (dd, *J* = 6.7, 4.7 Hz, 1H), 3.77 (dd, *J* = 4.6, 2.1 Hz, 1H), 3.67 (dd, *J* = 10.8, 3.3 Hz, 1H), 3.56 (dd, *J* = 10.8, 4.6 Hz ,1H). Spectral data are in accordance with literature.[28]

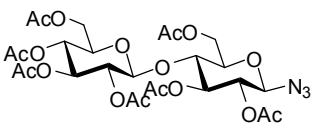
2-Acetamido-2-deoxy-β-D-glucopyranosyl azide (64)



To a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D glucopyranosyl azide (32, 100 mg, 0.27 mmol) in MeOH was added K_2CO_3 (5 mg, 0.03 mmol) and the mixture was stirred overnight. Next acidic ion–exchange (amberlite IR–120) was added until the solution became neutral. After filtration the

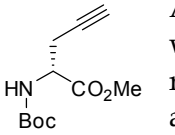
solvent was evaporated *in vacuo* to afford **64** (74 mg, 0.26 mmol, 96%) as a white solid. ^{1}H NMR (400 MHz, D_2O): δ = 4.72 (d, *J* = 9.1 Hz, 1H), 4.09−4.06 (m, 1H), 4.23−3.72 (m, 2H), 3.42−3.19 (m, 3H), 2.01 (s, 3H). Spectral data are in accordance with literature.[29]

2,3,5,2',3',4',6'-Hepta-*O*-acetyl-β-D-lactopyranosyl azide (73)



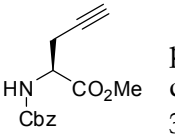
To a suspension of D-lactose (1.00 g, 2.92 mmol) in acetic anhydride (15 mL) was added NaOAc (240 mg, 2.93 mmol) and the resulting mixture was heated until reflux for 5 h. After cooling, EtOAc was added and the organic layer was washed with heptane, H_2O, saturated aqueous $NaHCO_3$ and brine, dried ($MgSO_4$) and evaporated *in vacuo* to afford the crude 1,2,3,5,2',3',4',6'–octa–*O*–acetylα,β–D–lactopyranose as slightly yellow solid. Next, trimethylsilyl azide (100 µL, 0.75 mmol) and tin tetrachloride (510 µL, 1M in DCM) were added at room temperature to a solution of the crude octaacetylated lactose (435 mg, 0.64 mmol) in dry DCM (4 mL). After 45 min, DCM was added and the solution was washed with H_2O, aqueous 1 M HCl and saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4), filtered and concentrated *in vacuo.* The crude product was purified by flash column chromatography (EtOAc/heptane, 1/1) to afford **73** (294 mg, 0.44 mmol, 69%) as a white solid. FTIR (ATR): ν = 2116, 1740, 1368, 1208 cm^{-1}. R_f = 0.48 (EtOAc/heptane, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.35 (dd, *J* = 3.4, 1.0 Hz, 1H), 5.20 (app t, *J* = 9.1 Hz, 1H), 5.10 (dd, *J* = 10.4, 7.9 Hz, 1H), 4.95 (dd, *J* = 10.4, 3.5 Hz, 1H), 4.86 (dd, *J* = 9.5, 8.8 Hz, 1H), 4.62 (d, *J* = 8.8 Hz ,1H), 4.52−4.47 (m, 2H), 4.15−4.05 (m, 3H), 3.88−3.79 (m, 2H), 3.70 (ddd, *J* = 9.9, 5.1, 2.1, 1H), 2.15 (s, 3H), 2.13 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 1.96 (s, 3H). Spectral data are in accordance with literature.[30]

Methyl (2*R*)-2-[(*tert*-butoxycarbonyl)amino]-4-pentynoate (25)



A suspension of (*R*)–propargylglycine (300 mg, 2.65 mmol) in MeOH (10 mL) was treated dropwise at 0 °C with $SOCl_2$ (0.5 mL, 6.85 mmol) and heated to reflux for 3 h. The reaction mixture was concentrated *in vacuo* to give the crude amino ester as the HCl-salt. The crude residue was suspended in DCM (20 mL), Et_3N (590 µL, 5.83 mmol) and Boc_2O (884.4 mg, 4.05 mmol) were added and the reaction mixture stirred for 3 h at room temperature. The mixture was concentrated *in vacuo* and purified by flash column chromatography (EtOAc/heptane, 3/1) to afford **25** (513 mg, 2.26 mmol, 85%). R*f* = 0.81 (EtOAc/heptane, 2/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.35 (d, *J* = 6.8 Hz, 1H), 4.51−4.46 (m, 1H), 3.78 (s, 3H), 2.77−2.71 (m, 2H), 2.05 (t, *J* = 2.5 Hz, 1H), 1.46 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.9, 154.8, 80.2, 78.4, 71.6, 52.8, 52.1, 28.5, 23.1. Spectral data are in accordance with literature.[20]

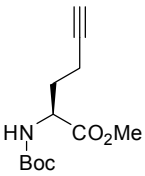
Methyl (2*S*)-[(benzyloxycarbonyl)amino]-4-pentynoate (42)



(*S*)-Propargylglycine (750 mg, 6.64 mmol) was dissolved in water (10 mL) at pH 11 (NaOH). Benzyl chloroformate (1.01 mL, 7.09 mmol) was added dropwise at 0 °C. A white precipitate of benzyl chloroformate was formed after 30 min. The reaction mixture was stirred overnight at ambient temperature. Next the pH was lowered to 1 using conc. aqueous H_2SO_4 and the product was extracted with EtOAc (3 ×). The combined organic layers were washed with brine, dried (Na_2SO_4) and evaporated *in vacuo* yielding 1.3 g (= 77%) of the crude Z–l–propargylglycine. (R_f = 0.72 (*n*–

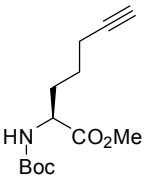
BuOH/HOAc/EtOAc/H_2O, 1/1/1/1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.67 (d, *J* = 8.4 Hz, 1H), 7.37−7.30 (m, 5H), 5.05 (d, 8.2 Hz, 2H), 4.16−4.12 (m, 1H), 2.87 (m, 1H), 2.60 (m, 2H)). Subsequently the crude Z–l–propargylglycine was dissolved in MeOH (10 mL) and $SOCl_2$ (0.4 mL, 5.51 mmol) was added at 0 °C. Next the solution was refluxed for 4 h, after which the solvent was evaporated *in vacuo*. The crude product was purified by flash column chromatography (EtOAc/heptanes, 1/1) to afford **42** (1.3 g, 4.98 mmol, 75% 2 steps). R*f* = 0.86 (EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.87 (d, *J* = 8.1 Hz, 1H), 7.40−7.29 (m, 5H), 5.05 (d, 8.2 Hz, 2H), 4.27−4.19 (m, 1H), 3.64 (s, 3H), 2.91−2.89 (m, 1H), 2.55−2.35 (m, 2H).

Methyl (2*S*)-2-[(*tert*–butoxycarbonyl)amino]-5-hexynoate (44)



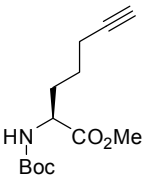
Applying the same procedure as for **25**, (*S*)-homopropargylglycine (65 mg, 0.51 mmol) was protected and purified by flash column chromatography to afford **44** (75 mg, 0.31 mmol, 61%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.22 (bd, *J* = 7.7 Hz, 1H), 4.41−4.37 (m, 1H), 3.75 (s, 3H), 2.28 (dt, *J* = 7.6, 2.6 Hz, 2H), 2.02−2.08 (m, 1H), 2.00 (t, *J* = 2.5 Hz, 1H), 1.92−1.87 (m, 1H), 1.45 (s, 9H). Spectral data are in accordance with literature.[20]

Methyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-6-heptynoate (46)



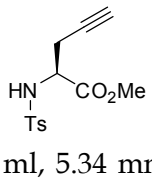
Applying the same procedure as for **25**, *(S)*-bishomopropargylglycine (148 mg, 1.05 mmol) was protected and purified by flash column chromatography to afford **46** (171 mg, 0.67 mmol, 64%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.05 (bd, *J* = 7.8 Hz, 1H), 4.33−4.30 (m, 1H), 3.75 (s, 3H), 2.23 (dt, *J* = 6.8, 2.6 Hz, 2H), 1.99 (t, *J* = 2.7 Hz, 1H), 1.88−1.51 (m, 4H), 1.45 (s, 9H). Spectral data are in accordance with literature.[20]

(2*S*)-2-[(9*H*-Fluoren-9-ylmethoxycarbonyl)amino]-4-pentynoic acid (50)



A mixture of (*S*)-propargylglycine (102 mg, 0.91 mmol) and Na_2CO_3 (107 mg, 1.01 mmol) in H_2O (1.5 mL) was added dropwise to a stirred solution of Fmoc–succinimide in dioxane, cooled in ice to 8 °C. The solution was allowed to warm to room temperature overnight and was poured into water after 24 h. The solution was extracted with DCM (2 ×) and the organic fractions were combined, washed, dried ($MgSO_4$) and concentrated *in vacuo*. Recrystallization from Et_2O/hexane afforded **50** (159 mg, 0.48 mmol, 57%). R_f = 0.17 (EtOAc/heptane, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 7.6 Hz, 2H), 7.61 (d, *J* = 7.2 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.5 Hz, 2H), 5.69 (d, *J* = 7.8 Hz, 1H), 4.62−4.50 (m, 1H), 4.41 (d, *J* = 7.0 Hz, 2H), 4.25 (t, *J* = 7.0 Hz, 1H), 2.84 (br s, 2H), 2.12 (br s, 1H).

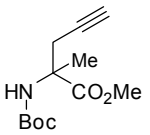
Methyl (2*S*)-(4-methylphenylsulfonamido)pent-4-ynoate (52)



A suspension of (*S*)-propargylglycine (202 mg, 1.78 mmol) in MeOH (10 mL) was treated dropwise at 0 °C with $SOCl_2$ (0.15 mL, 2.05 mmol) and refluxed for 3 h. The reaction mixture was concentrated *in vacuo* to give the crude amino ester HCl-salt. The crude residue was suspended in DCM (10 mL), Et_3N (0.75 ml, 5.34 mmol) and *p*-TsCl (411 mg, 2.16 mmol) were added and the reaction mixture was stirred for 4 h. The mixture was concentrated *in vacuo* and taken up in EtOAc and H_2O. The aqueous layer was extracted with EtOAc (3 ×) and the combined organic layers were washed

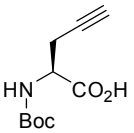
with brine, dried (Na_2SO_4) and concentrated *in vacuo*. The crude product was purified by flash column chromatography (EtOAc/heptane, 1/1) to afford **52** (107 mg, 0.38 mmol, 21%) R_f = 0.83 (EtOAc/heptane, 2/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.75 (d, *J* = 8.3 Hz, 2H), 7.30 (d, *J* = 8.3 Hz, 2H), 5.50 (d, *J* = 8.6 Hz, 1H), 4.14−4.10 (m, 1H), 3.61 (s, 3H), 2.74−2.61 (m, 2H), 2.42 (s, 3H), 2.03 (t, *J* = 2.6 Hz, 1H). Spectral data are in accordance with literature.[20]

Methyl 2-(*tert*-butoxycarbonylamino)-2-methyl-4-pentynoate (56)



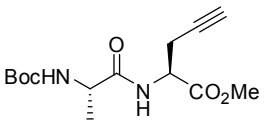
Applying the same procedure as for **25**, α-methylhomopropargylglycine (300 mg, 2.36 mmol) was protected and purified by flash column chromatography to afford **56** (310 mg, 1.28 mmol, 54%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.23 (bs, 1H), 3.76 (s, 3H), 2.96−2.86 (m, 2H), 2.02 (t, *J* = 2.7 Hz, 1H), 1.55 (s, 3H), 1.43 (s, 9H).

(2*S*)-2-[(*tert*-Butoxycarbonyl)amino]-4-pentynoic acid (62)



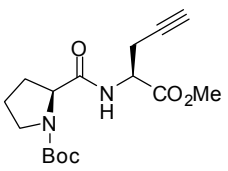
To a suspension of (*S*)-propargylglycine (145 mg, 1.29 mmol) in dioxane/water (20 mL, 1/1) was added Boc_2O (375 mg, 1.72 mmol) followed by a 2M aqueous NaOH solution (1.3 mL). The mixture was stirred until completion (TLC) after which DCM (3 mL) and $KHSO_4$ (3 mL, 5% solution in water) were added. The product was extracted with DCM (3 ×) and the combined organic layers were washed with brine, dried ($MgSO_4$) and evaporated *in vacuo* to afford **62** as a white solid (248 mg, 90%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.34 (br d, *J* = 7.5 Hz, 1H), 4.53−4.49 (m, 1H), 2.85−2.73 (m, 2H), 2.08 (t, 1H), 1.47 (s, 9H).

2-(2-*tert*-Butoxycarbonylaminopropionylamino)-pent-4-ynoic acid methyl ester (67).



A suspension of (*S*)-propargylglycine (308.9 mg, 2.73 mmol) in MeOH (10 mL) was treated dropwise at 0 °C with $SOCl_2$ (2.5 mL, 3.45 mmol) and refluxed for 3 h. The reaction mixture was concentrated *in vacuo* affording the crude amino ester as the HCl–salt. A part of the crude residue (174 mg, 1.37 mmol) was dissolved in DCM (5 mL), Et_3N (0.23 mL, 1.63 mmol), Boc-L-alanine (266 mg, 1.41 mmol) and PyBOP (849.2 mg, 1.63 mmol) were added, the reaction mixture was kept basic by adding Et_3N (0.35 mL, 2.49 mmol), and stirred for 4 h. Water was added and the aqueous solution was extracted with DCM (3 ×). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. Purification by flash column chromatography (EtOAc/heptane, 1/2) afforded **67** (144 mg, 0.48 mmol, 35%) as a white solid. R_f = 0.36 (EtOAc/heptane, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.86 (d, *J* = 6.1 Hz, 1H), 4.97 (br s, 1H), 4.72 (dt, *J* = 7.8, 4.7 Hz, 1H), 4.28−4.16 (m, 1H), 3.79 (s, 3H), 2.80−2.75 (m, 2H), 2.02 (t, *J* = 2.7 Hz, 1H), 1.46 (s, 9H), 1.39 (d, *J* = 7.0 Hz, 3H).

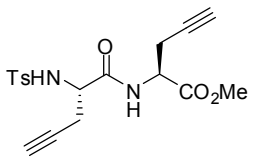
2-(1-Methoxycarbonylbut-3-ynylcarbamoyl)pyrrolidine-1-carboxylic acid *tert*-butyl ester (69).



A suspension of (*S*)-propargylglycine (309 mg, 2.73 mmol) in MeOH (10 mL) was treated dropwise at 0 °C with $SOCl_2$ (2.5 mL, 3.45 mmol) and refluxed for 3 h. The reaction mixture was concentrated *in vacuo* affording the crude amino ester as the HCl–salt. A part of the crude residue (173.5 mg, 1.37 mmol) was dissolved in DCM (5 mL), Et_3N (0.23 mL, 1.63 mmol),

Boc-L-proline (292 mg, 1.36 mmol) and PyBOP (869 mg, 1.67 mmol) were added, the reaction mixture was kept basic by adding Et_3N (0.35 mL, 2.49 mmol), and stirred for 4 h. Water was added and the aqueous solution was extracted with DCM (3 ×). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. Purification by flash column chromatography (EtOAc/heptane, 1/1) afforded **69** (309 mg, 0.95 mmol, 70%) as a white solid. R_f = 0.45 (EtOAc/heptane, 2/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.72 (br. s, 1H), 4.40−4.22 (m, 1H), 3.78 (s, 3H), 3.57−3.27 (m, 2H), 2.83−2.69 (m, 2H), 2.38−2.10 (m, 2H), 2.00 (br. s, 1H), 1.96−1.84 (m, 2H), 1.48 (s, 9H).

(*S*)-Methyl 2-((*S*)-2-(4-methylphenylsulfonamido)pent-4-ynamido)pent-4-ynoate (71).

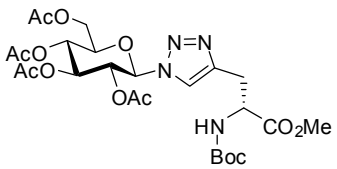


This product was formed as byproduct when synthesizing methyl (2*S*)-2-{[(4-methylphenyl)sulfonyl]amino}-4-pentynoate (**52**). R_f = 0.61 (EtOAc/heptane, 2/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.77 (app d, *J* = 8.3 Hz, 2H), 7.34−7.31 (m, 2H), 7.24 (bd, *J* = 8.5 Hz, 1H), 5.46 (d, *J* = 8.1 Hz, 1H), 4.63 (t, *J* = 4.7 Hz, 1H), 4.61 (t, *J* = 4.7, 1H), 3.91 (ddd, *J* = 8.1, 7.1, 4.6 Hz, 1H), 3.79 (s, 3H), 2.80 (ddd, *J* = 17.0, 4.6, 2.7 Hz, 1H), 2.72 (ddd, *J* = 17.0, 4.4, 2.7 Hz, 1H), 2.60 (ddd, *J* = 17.0, 5.0, 2.7 Hz, 1H), 2,43 (s, 3H), 2.37 (ddd, *J* = 17.0, 7.0, 2.7 Hz, 1H), 2.07 (t, *J* = 2.6, 1H), 2.02 (t, *J* = 2.6, 1H).

General procedure A for the cycloaddition:

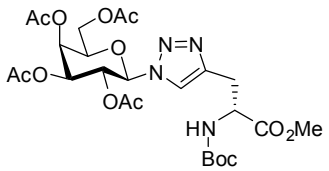
To a solution of the glycoside (1 equiv) and the amino acid derivative (1 equiv) in *tert*–butanol (0.5 M) was added a mixture of $Cu(OAc)_2$ (20 mol%) and sodium ascorbate (40 mol%) in H_2O (0.04 M and 0.08 M, respectively). The reaction was stirred overnight, water was added and the product was extracted with DCM (2 ×). The combined organic layers were washed with saturated aqueous $NaHCO_3$ (except for the free acids), brine, dried ($NaSO_4$) and evaporated *in vacuo*. The product was purified by flash column chromatography using EtOAc/heptane mixtures.

Boc-(*R*)-T4M(1-[β-D-Glc(Ac)$_4$])-OMe (27)



Preparation according to general procedure A for the cycloaddition afforded **27** (232 mg, 0.39 mmol, 98%) as a white solid. R_f = 0.37 (EtOAc/heptane, 2/1). $[\alpha]_D^{20}$ = −32.8 (c = 0.69, DCM). FTIR (ATR): ν = 3391, 2980, 2362, 2340, 1738, 1709, 1498 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.60 (s, 1H), 5.83−5.80 (m, 1H), 5.47−5.38 (m, 3H), 5.26−5.21 (m, 1H), 4.64−4.62 (m,1H), 4.31 (dd, *J* = 12.6, 5.0 Hz, 1H), 4.15 (dd, *J* = 12.6, 2.0 Hz, 1H), 3.99 (ddd *J* = 10.1, 5.0, 2.1, 1H), 3.73 (s, 3H), 3.28 (dd, *J* = 15.0, 5.3, 1H) 3.20 (dd, *J* = 15.0, 4.6 Hz 1H), 2.09 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 1.89 (s, 3H), 1.45 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.4, 170.2, 169.6, 169.1, 168.8, 155.2, 143.4, 120.6, 85.8, 80.0, 75.3, 72.6, 70.4, 67.9, 61.7, 53.1, 52.6, 28.8, 28.6, 21.0, 20.9, 20.8, 20.4. 1455. HRMS (ESI) *m/z* calculated for $C_{25}H_{37}O_{13}N_4$ $(M+H)^+$: 601.2357, found: 601.2383.

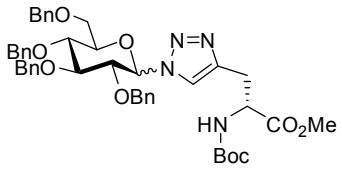
Boc-(*R*)-T4M(1-[β-D-Gal(Ac)$_4$])-OMe (29)



Preparation according to general procedure A for the cycloaddition afforded **29** (44 mg, 0.07 mmol, 89%) as a white solid. R_f = 0.32 (EtOAc/heptane, 2/1). $[\alpha]_D^{20}$ = −14.1 (c = 1.41,

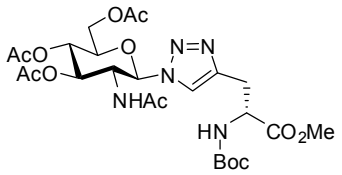
DCM). FTIR (ATR): ν = 3373, 2976, 2358, 2332, 1753, 1709 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.93 (s, 1H), 5.78 (d, *J* = 9.3 Hz, 1H), 5.59−5.42 (m, 3H), 5.24 (dd, *J* = 10.4, 3.3 Hz, 1H), 4.67−4.59 (m, 1H), 4.24−4.10 (m, 3H), 3.73 (s, 3H), 3.29 (dd, *J* = 15.1, 5.2 Hz, 1H), 3.21 (dd, *J* = 15.1, 4.6 Hz, 1H), 2.23 (s, 3H), 2.05 (s, 3H), 2.01 (s, 3H), 1.90 (s, 3H), 1.45 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.5, 170.1, 169.7, 169.6, 169.0, 155.2, 143.4, 120.8, 86.4, 80.0, 74.3, 70.8, 68.0, 67.1, 61.3, 53.2, 52.7, 30.0, 28.6, 21.0, 20.8, 20.5. HRMS (ESI) *m/z* calculated for $C_{25}H_{37}O_{13}N_4$ $(M+H)^+$: 601.2357, found: 601.2347.

Boc-(*R*)-T4M(1-[D-Glc(Bn)$_4$])-OMe (30a/b)



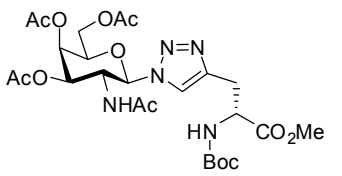
Preparation according to general procedure A for the cycloaddition afforded **30a** (64 mg, 0.08 mmol, 87%) and **30a/b** (58 mg, 0.07 mmol, 87%) as a white solid. R_f = 0.47 (EtOAc). Spectral data of major isomer (**30a**): FTIR (ATR): ν = 3391, 3028, 2915, 2362, 2323, 1744, 1714, 1498 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.46 (s, 1H), 7.36−7.27 (m, 13H), 7.24−7.20 (m, 3H), 7.19−7.13 (m, 3H), 6.99−6.94 (m, 2H), 4.93 (d, *J* = 11.1 Hz, 1H), 4.89 (d, *J* = 10.9 Hz, 1H), 4.85 (d, *J* = 10.7 Hz, 1H), 4.60 (d, *J* = 10.8 Hz, 1H), 4.54 (d, *J* = 12.1, 1H), 4.48 (d, *J* = 12.1 Hz, 1H), 4.47 (d, *J* = 10.6 Hz, 1H), 4.07−3.96 (m, 1H), 4.02 (d, *J* = 10.5 Hz, 1H), 3.90−3.76 (m, 2H), 3.74−3.65 (m, 3H), 3.64 (s, 3H), 3.24 (d, *J* = 4.5 Hz, 2H), 1.41 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.6, 155.2, 143.0, 138,0, 137.7, 136.9, 128.4, 128.4, 128.3, 128.2, 127.9, 127.9, 127.8, 127.7, 127.7, 121.4, 87.6, 85.5, 81.0, 80.2, 78.2, 77.4, 75.9, 75.4, 75.1, 73.7, 68.6, 53.2, 52.7, 28.6. HRMS (ESI) *m/z* calculated for $C_{45}H_{53}O_{19}N_4$ $(M+H)^+$: 793.3812, found: 793.3837

Boc-(*R*)-T4M(1-[β-D-GlcNAc(Ac)$_3$])-OMe (33)



Preparation according to general procedure A for the cycloaddition afforded **33** (70 mg, 0.12 mmol, 91%) as a white solid. R_f = 0.44 (EtOAc). $[\alpha]_D^{20}$ = −44.5 (c = 0.91, DCM). FTIR (ATR): ν = 3304, 2976, 2250, 1753 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ =7.69 (s, 1H), 6.2 (br d, *J* = 9.0 Hz, 1H), 5.93 (d, *J* = 10.0 Hz, 1H), 5.65 (d, *J* = 8.6 Hz, 1H), 5.45 (dd, *J* = 10.0, 9.8 Hz, 1H), 5.21 (dd, *J* = 9.8, 9.6 Hz, 1H), 4.65−4.52 (m, 2H), 4.27 (dd, *J* = 12.6, 4.7 Hz, 1H), 4.13 (dd, *J* = 12.6, 2.1 Hz, 1H), 4.03 (ddd, *J* = 10.1, 4.7, 2.1 Hz, 1H), 3.71 (s, 3H), 3.28 (dd, *J* = 14.8, 5.7, 1H), 3.16 (dd, *J* = 14.8, 4.9, 1H), 2.07 (s, 3H), 2.06 (s, 6H), 1.77 (s, 3H), 1.43 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.6, 170.7, 170.4, 170.3, 169.1, 155.4, 142.9, 121.7, 86.2, 80.0, 75.1, 72.3, 68.2, 61.9, 53.7, 53.3, 52.6, 28.8, 28.6, 23.0, 21.0, 21.0, 20.9. HRMS (ESI) *m/z* calculated for $C_{25}H_{37}O_{12}N_5$ $(M+H)^+$: 622.2336, found: 622.2367.

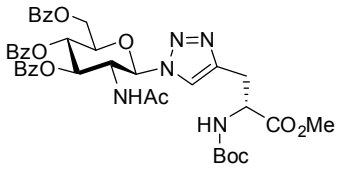
Boc-(*R*)-T4M(1-[β-D-GalNAc(Ac)$_3$])-OMe (35)



Preparation according to general procedure A for the cycloaddition afforded **35** (17.1 mg, 0.03 mmol, 72%) as a white solid. R_f = 0.41 (EtOAc). $[\alpha]_D^{20}$ = −22.0 (c = 0.58, DCM). FTIR (ATR): ν = 3321, 2980, 2353, 2340, 1748 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.72 (s, 1H), 5.90−5.87 (m, 2H), 5.68 (br d, *J* = 8.6 Hz, 1H), 5.50 (d, *J* = 2.7 Hz, 1H), 5.45−5.34 (m, 1H), 4.75−4.59 (m, 2H), 4.27−4.04 (m, 3H), 3.73 (s, 3H), 3.30 (dd, *J* = 14.5, 4.9 Hz, 2H), 3.17 (dd, *J* = 14.5, 4.4 Hz, 1H), 2.22 (s, 3H), 2.03 (s, 3H),

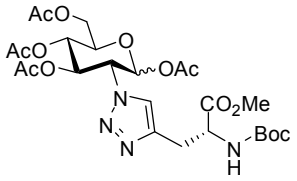
2.03 (s, 3H), 1.79 (s, 3H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.6, 170.6, 170.2, 169.9, 155.5, 142.8, 121.9, 86.7, 79.9, 74.1, 72.0, 66.8, 61.6, 53.3, 52.6, 50.4, 28.7, 23.2, 21.0. HRMS (ESI) *m/z* calculated for $C_{25}H_{37}O_{12}N_5$ $(M+H)^+$: 622.2336, found: 622.2383.

Boc-(*R*)-T4M(1-[β-D-GlcNAc(Bz)$_3$])-OMe (37)



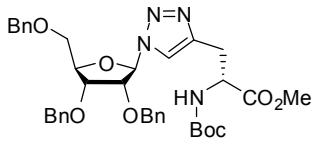
Preparation according to general procedure A for the cycloaddition afforded **37** (23 mg, 0.03 mmol, 30%) as a white solid. R_f = 0.33 (EtOAc/heptane, 1/1). $[\alpha]_D^{20}$ = +5.0 (c = 1.38, DCM). FTIR (ATR): ν = 3321, 2980, 2353, 2340, 1748 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.01 (d, *J* = 6.8 Hz, 2H), 7.94−7.78 (m,4H), 7.73 (s, 1H), 7.53−7.32 (m, 9H), 6.05 (d, *J* = 9.6 Hz, 1H), 5.89−5.80 (m, 3H), 5.70−5.69 (m, 1H), 4.84−4.79 (m, 1H), 4.67−4.62 (m, 1H), 4.64 (dd, *J* = 12.4, 2.8 Hz, 1H), 4.47 (dd, *J* = 12.4, 4.8 Hz, 1H), 4.36 (ddd, *J* = 9.2 ,4.8, 2.4, 1H), 3.70 (s, 3H), 3.32 (dd, *J* = 14.4, 5.4, 1 H), 1.72 (s, 3H), 1.42 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.9, 167.0, 166.3, 165.2, 143.4, 134.2, 134.0, 133.7, 130.3, 130.2, 130.1, 129.7, 129.0, 128.9, 128.8, 128.6, 122.2, 87.2, 77.8, 76.0, 73.3, 69.3, 63.3, 54.8, 53.7, 53.0, 29.1, 23.5. HRMS (ESI) *m/z* calculated for $C_{40}H_{44}N_5O_{12}$ $(M+H)^+$: 786.2987, found: 786.3059.

Boc-(*R*)-T4M(1-[2-deoxy-1,3,4,6-tetra-*O*-acetyl-α,β-D-glucopyranos-2-yl])-OMe (39)



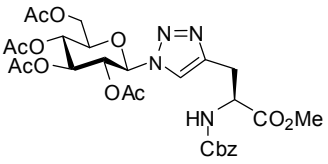
Preparation according to general procedure A for the cycloaddition afforded **39** (43 mg, 0.07 mmol, 71%) as a white solid. R_f = 0.30 (EtOAc/heptane, 2/1). Major isomer: FTIR (ATR): ν = 3382, 2980, 2358, 2336, 2250, 1744 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.39 (s, 3H), 6.13 (d, *J* = 8.6 Hz, 1H), 5.75−5.70 (m, 1H), 5.46 (br d, *J* = 7.6 Hz, 1H) 5.26−5.09 (m, 1H), 4.63 (dd, *J* = 10.6, 8.9 Hz, 1H), 4.59−4.51 (m, 1H), 4.34 (dd, *J* = 12.4, 4.4 Hz, 1H), 4.13 (dd, *J* = 12.4, 1.6 Hz, 1H), 4.05−4.01 (m, 1H), 3.66 (s, 3H), 3.18 (br d, *J* = 4.1 Hz, 2H), 2.06 (s, 3H), 2.00 (s, 3H), 1.96 (s, 3H), 1.84 (s, 3H), 1.39 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.4, 170.3, 169.3, 169.0, 168.0, 155.1, 142.9, 122.1, 91.6, 80.1, 73.2, 72.0, 68.1, 62.8, 61.5, 53.1, 52.6, 28.6, 21.0, 20.8, 20.7, 20.5. HRMS (ESI) calculated for $C_{25}H_{36}N_4O_{13}Na$ $(M+Na)^+$: 623.2177, found: 623.2180.

Boc-(*R*)-T4M(1-[β-D-Ara(Bn)$_3$])-OMe (41)



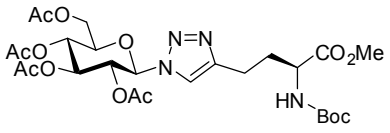
Preparation according to general procedure A for the cycloaddition afforded **41** (60mg, 0.09 mmol, 88%) as a white solid. R_f = 0.49 (EtOAc/heptane, 1/1). FTIR (ATR): ν = 3369, 1735, 1709, 1217 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.58 (s, 1H), 7.37−7.21 (m, 15 H), 6.16 (d, *J* = 2.8 Hz, 1H), 5.51 (d, *J* = 8.2 Hz, 1H), 4.69−4.44 (m, 6H), 4.42 (dd, *J* = 6.4, 3.2 Hz, 1H),4.39 (dd, *J* = 6.4, 3.2 Hz, 1H), 4.18 (dd, *J* = 6.4, 4.8 Hz, 1H), 3.78 (dd, *J* = 10.9, 2.9 Hz, 1H), 3.68 (s, 3H), 3.58 (dd, *J* = 10.9, 3.3 Hz, 1H), 3.07−2.96 (m, 2H), 1.42 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.5, 155.0, 142.2, 137.1, 136.9, 136.5, 128.0, 127.7, 127.6, 127.5, 127.3, 120.6, 90.8, 81.7, 80.0, 79.4, 75.6, 73.0, 72.1, 68.4, 52.5, 51.9, 27.8. HRMS (ESI) *m/z* calculated for $C_{37}H_{45}O_8N_4$ $(M+H)^+$: 673.3237, found: 673.3260 and HRMS (ESI) *m/z* calculated for $C_{37}H_{44}O_8N_4$ $(M+Na)^+$: 695.3057, found: 695.3066.

Cbz-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-OMe (43)



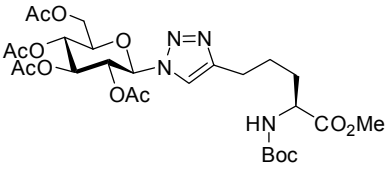
Preparation according to general procedure A for the cycloaddition afforded **43** (50%) as a white solid. FTIR (ATR): ν = 3373, 2950, 1740, 1212 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.55 (s, 1H), 7.38−7.28 (m, 5H), 5.82 (d, *J* = 9.1 Hz, 2H), 5.44−5.30 (m, 2H), 5.21 (dd, *J* = 10.1, 9.2 Hz, 1H), 5.09 (s, 2H), 4.75−4.65 (m, 1H), 4.28 (dd, *J* = 12.6, 5.0 Hz, 1H), 4.12 (dd, *J* = 12.6, 2.1 Hz, 1H), 3.98 (ddd, *J* = 10.1, 5.0, 2.1 Hz, 1H), 3.73 (s, 3H), 3.27 (d, *J* = 4.9 Hz, 2H), 2.05 (s, 3H), 2.05 (s, 3H), 2.00 (s, 3H), 1.81 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.5, 170.5, 169.9, 169.4, 169.2, 156.0, 143.5, 136.4, 128.6, 128.2, 120.5, 85.8, 75.2, 72.6, 70.3, 67.8, 67.1, 61.6, 53.3, 52.7, 28.1, 20.8, 20.6, 20.6, 20.1. HRMS (ESI) *m/z* calculated for $C_{28}H_{34}N_4O_{13}Na$ $(M^+Na)^+$: 657.2020, found: 657.2025.

Boc-(*S*)-T4E(1-[β-D-Glc(Ac)$_4$])-OMe (45)



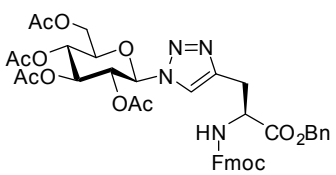
Preparation according to general procedure A for the cycloaddition afforded **45** (50 mg, 0.08 mmol, 80%) as a white solid. R_f = 0.29 (EtOAc/heptane, 2/1). $[\alpha]_D^{20}$ = −28.4 (c = 0.89, DCM). FTIR (ATR): ν 3382, 2980, 1753, 1710 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.57 (s, 1H), 5.86−5.80 (m, 1H), 5.44−5.36 (m, 2H), 5.26−5.19 (m, 1H), 5.17, (br d, *J* = 8.6 Hz, 1H), 4.31 (d, *J* = 5.1 Hz ,1H), 4.28 (dd, *J* = 12.7, 4.9 Hz, 1H), 4.13 (dd, *J* = 12.7, 2.0 Hz, 1H), 3.98 (ddd, *J* = 10.1, 4.9, 2.0 Hz, 1H), 3.71 (s, 3H), 2.78 (t, *J* = 8.0 Hz, 2H), 2.24−2.16 (m, 1H), 2.06 (s, 3H), 2.05 (s, 3H), 2.03−1.95 (m, 1H), 2.00 (s, 3H), 1.85, (s, 3H), 1.42 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.6, 170.2, 169.7, 169.1, 168.7, 155.2, 147.2, 119.4, 85.7, 80.1, 75.2, 72.8, 70.3, 67.9, 61.7, 53.1, 52.6, 32.5, 28.6, 22.1, 21.0, 20.9, 20.8, 20.5. HRMS (ESI) *m/z* calculated for $C_{26}H_{38}N_4O_{13}Na$ $(M+Na)^+$: 637.2333, found: 637.2374.

Boc-(*S*)-T4P(1-[β-D-Glc(Ac)$_4$])-OMe (47)



Preparation according to general procedure A for the cycloaddition afforded **47** (45 mg, 0.07 mmol, 76%) as a white solid. R_f = 0.29 (EtOAc/heptane, 2/1). Major isomer: $[\alpha]_D^{20}$ = −11.1 (c = 0.76, DCM). FTIR (ATR): ν = 3378, 2958, 1744, 1714 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.54 (s, 1H), 5.85 (d, *J* = 8.8 Hz, 1H), 5.45−5.36 (m, 2H), 5.24 (dd, *J* = 9.8, 9.4 Hz, 1H), 5.15 (br d, *J* = 8.2 Hz, 1H), 4.35−4.26 (m, 1H), 4.32 (dd, *J* = 12.7, 5.0 Hz, 1H) 4.15 (dd, *J* = 12.7, 2.0 Hz, 1H), 4.01 (ddd, *J* = 10.2, 4.9, 2.0 Hz, 1H), 3.74 (s, 3H), 2.84−2.65 (m, 2H), 2.09 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 1.80 (s, 3H), 1.88−1.57 (m, 4H), 1.43 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.1, 170.4, 169.8, 169.3, 169.0, 155.5, 148.2, 119.3, 86.0, 80.1, 75.4, 72.8, 70.8, 68.1, 61.9, 53.6, 52.7, 32.3, 28.8, 25.7, 25.5, 21.2, 21.0, 21.0, 20.6. HRMS (ESI) *m/z* calculated for $C_{27}H_{40}N_4O_{13}Na$ $(M+Na)^+$: 651.2490, found: 651.2521.

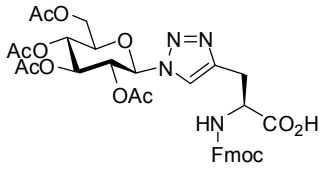
Fmoc-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-OBn (49)



Preparation according to general procedure A for the cycloaddition afforded **49** (83.4 mg, 0.11 mmol, 70%) as a white solid. R_f = 0.16 (EtOAc/heptane, 1/1). $[\alpha]_D^{20}$ = −5.7 (c = 0.29, DCM). FTIR (ATR): ν = 3378, 2950, 2353, 2340, 1753 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.03−7.89 (m, 5H), 8.09 (d, *J* = 7.4

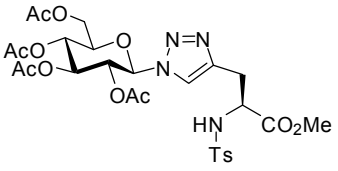
Hz, 2H), 7.73 (s, 1H), 7.58−7.48 (m, 4H), 7.44−7.32 (m, 7H), 6.05 (d, *J* = 9.7 Hz, 1H), 5.88−5.77 (m, 3H), 5.70−5.67 (m, 1H), 4.84−4.78 (m, 1H), 4.64 (dd, *J* = 12.4, 2.6 Hz, 2H), 4.47 (dd, *J* = 12.4, 4.8 Hz, 2H), 4.39−4.34 (m, 1H), 3.70 (s, 3H), 3.23 (dd, *J* = 14.8, 5.1 Hz, 1H), 3.16 (dd, *J* = 14.8, 4.4 Hz, 1H), 1.73 (s, 3H), 1.42 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.6, 170.2, 169.6, 169.2, 169.0, 155.8, 143.8, 143.7, 141.1, 135.3, 128.6, 128.5, 127.6, 127.0, 125.2, 120.5, 120.0, 85.9, 73.4, 72.5, 70.6, 67.9, 67.6, 67.4, 61.7, 53.6, 47.4, 28.4, 21.0, 20.9, 20.9, 20.4. HRMS (ESI) *m/z* calculated for $C_{41}H_{42}N_4O_{13}Na$ $(M+Na)^+$: 821.2646, found: 821.2674.

Fmoc-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-OH (51)



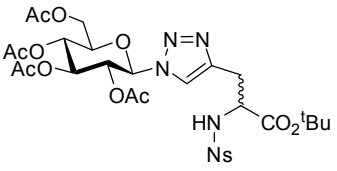
Preparation according to general procedure A for the cycloaddition afforded **51** (3.24g, 4.59 mmol, 91%) as a white solid. R_f = 0.10 (EtOAc/heptane 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.75 (d, *J* = 7.6 Hz, 1H), 7.70 (s, 1H), 7.61−7.58 (m, 2H), 7.41−7.37 (m, 2H), 7.32−7.28 (m, 2H), 6.00 (d, *J* = 7.8 Hz, 1H), 5.86−5.83 (m, 1H), 5.48−5.35 (m, 2H), 5.31−5.19 (m, 1H), 4.80−4.67 (m, 1H), 4.38 (d, *J* = 7.2 Hz, 2H), 4.30 (ABdd, *J* = 12.6, 5.1 Hz, 1H), 4.23 (t, *J* = 7.2 Hz, 1H), 4.15−4.09 (m, 1H), 3.99 (ddd, *J* = 10.2, 4.9, 2.0 Hz, 1H), 3.45−3.30 (m, 2H), 2.10 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 1.84 (s, 3H). ^{13}C NMR (75 M Hz, $CDCL_3$): δ = 173.2, 170.7, 170.0, 169.8, 169.6, 156.2, 143.9, 143.6, 141.4, 127.9, 127.2, 125.3, 121.4, 120.1, 85.9, 75.4, 72.6, 70.6, 67.9, 67.4, 61.7, 53.4, 47.3, 27.9, 20.8, 20.7, 20.6, 20.2. HRMS (ESI) *m/z* calculated for $C_{34}H_{37}N_4O_{13}$ $(M+H)^+$: 709.2344 found: 709.2357

Ts-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-OMe (53)



Preparation according to general procedure A for the cycloaddition afforded **53** (60 mg, 0.09 mmol, 64%) as a white solid. R_f = 0.28 (EtOAc/heptane, 2/1). $[\alpha]_D^{20}$ = −8.6 (c = 0.40, DCM). FTIR (ATR): ν = 3481, 3278, 3140, 2950, 2362, 2254, 1744, 1601v. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.73 (d, *J* = 8.2 Hz, 2H), 7.60 (s, 1H), 7.29 (d, *J* = 8.0 Hz, 2H), 5.83 (d, *J* = 9.0 Hz, 1H), 5.57 (d, *J* = 8.8 Hz, 1H), 5.42 (t, *J* = 9.3 Hz, 1H), 5.38 (t, *J* = 9.2 Hz, 1H), 5.24 (dd, *J* = 10.0, 9.2 Hz, 1H), 4.32 (dd, *J* = 12.7, 4.9 Hz, 1H), 4.284.26 (m, 1H), 4.16 (dd, *J* = 12.7, 2.0 Hz, 1H), 4.00 (ddd, *J* = 10.0, 6.8, 2.0 Hz, 1H), 3.57 (s, 3H), 3.19 (d, *J* = 5.2 Hz, 2H), 2.42 (s, 3H), 2.10 (s, 3H), 2.07 (s, 3H), 2.07 (s, 3H), 1.89 (s, 3H), 1.58 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.5, 170.3, 169.7, 169.1, 169.1, 143.5, 142.5, 136.8, 129.6, 127.2, 120.9, 85.9, 75.3, 72.7, 70.5, 67.9, 61.7, 54.9, 53.0, 29.7, 21.9, 21.1, 20.9, 20.9, 20.5. HRMS (ESI) *m/z* calculated for $C_{27}H_{34}O_{13}N_4NaS$ $(M+Na)^+$: 677.1741, found: 677.1767.

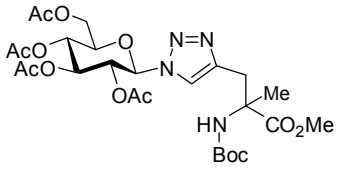
Ns-T4M(1-[β-D-Glc(Ac)$_4$])-O^{t}Bu (55)



Preparation according to general procedure A for the cycloaddition afforded **55** (66 mg, 0.09 mmol, 87%) as a mixture of isomers (1:1). R_f = 0.26 (EtOAc/heptane, 1/1). FTIR (ATR): ν = 3369, 1735, 1709, 1221 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.31 & 8.29 (ap d, *J* = 9.1 Hz, 2H), 8.02 & 8.00 (ap d, *J* = 9.1 Hz, 2H), 9.79 & 7.61 (s, 1H), 6.01 & 5.91 (d, *J* = 9.7 & 9.0 Hz, 1H), 5.83 & 5,78 (d, *J* = 9.1 & 8.9 Hz, 1H), 5.47−5.33 (m, 2H), 5.24 (dd, *J* = 19.4, 9.3 Hz, 1H), 4.31 & 4.28 (dd, *J* = 12.7, 4.9 Hz, 1H), 4.24−4.20 (m, 1H), 4.19−4.14 (m, 1H), 4.02 & 4.01 (ddd, *J* = 7.5, 5.0, 1.9 & 6.5, 5.2, 1.9, 1H), 3.35 & 3.25 (ABdd, *J* = 14.6, 4.1 & 15.3, 5.3, 1H), 3.20 & 3.14 (ABdd, *J* = 14.6, 4.7 & 15.3, 4.8, 1H), 2.09 (s, 3H), 2.06 (s, 3H), 2.06 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H) 2.02 (s, 3H), 1.98 (s, 3H), 1.86 (s,

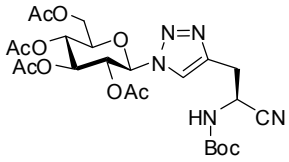
3H), 1.27 (s, 9H), 1.26 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.3, 170.2, 170.0, 169.6, 169.6, 169.3, 169.0, 169. 168.9, 167.9, 149.9, 149.8, 145.9, 145.9, 142.5, 142.0, 128.4, 124.1, 124.0, 121.7, 120.7, 85.9, 85.8, 83.5, 83.4, 75.4, 75.3, 72.5, 72.1, 70.9, 70.7, 68.0, 67.8, 61.7, 61.6, 60.6, 55.6, 55.6, 30.1, 29.9, 28.0, 28.0, 21.0, 21.0, 20.9, 21.8, 20.5, 20.4, 14.5, 14.5. HRMS (ESI) *m/z* calculated for $(C_{29}H_{37}O_{15}N_5)_2Na$ $(2M+Na)^+$: 1477.3911, found: 1477.3820.

Boc-α-methyl-T4M(1-[β-D-Glc(Ac)$_4$])-OMe (57)



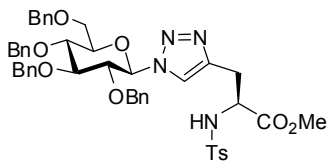
Preparation according to general procedure A for the cycloaddition afforded **57** (55 mg, 0.09 mmol, 85%) as a white solid. R_f = 0.15 (EtOAc/heptane, 2/1). Mixture of diastereoisomers: FTIR (ATR): ν = 3382, 2958, 2362, 1748, 1709 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.52 (s, 1H), 7.51 (s, 1H), 5.82 (d, *J* = 9.2 Hz, 1H), 5.81 (d, *J* = 9.4 Hz, 1H), 5.43−5.27 (m, 6H), 5.21 (t, *J* = 10.3 Hz, 2H), 4.28 (dd, *J* = 12.5, 5.0 Hz, 2H), 4.12 (dd, *J* = 12.5, 2.0 Hz, 2H), 4.01−3.97 (m, 4H), 2.05 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H), 1.84 (s, 3H), 1.84 (s, 3H), 1.42 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.8, 173.8, 170.2, 170.2, 169.6, 169.6, 169.1, 169.1, 168.7, 168.6, 154.2, 154.2, 143.6, 143.6, 121.0, 121.0, 85.8, 85.8, 79.9, 75.3, 75.2, 72.6, 72.5, 70.7, 70.5, 67.9, 67.9, 61.7, 61.7, 59.3, 59.2, 52.9, 52.8, 28.6, 28.6, 23.8, 23.7, 21.0, 20.8, 20.8, 20.4, 20.3. HRMS (ESI) *m/z* calculated for $C_{26}H_{38}N_4O_{13}Na$ $(M+Na)^+$: 637.2333, found: 637.2352.

(*S*)-2-[(*tert*-Butoxycarbonyl)amino]-3-{1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)[1,2,3]triazol-4-yl}propanenitrile(59).



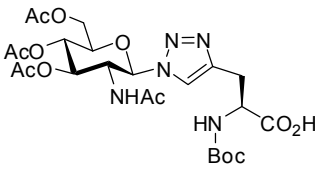
Preparation according to general procedure A for the cycloaddition afforded **59** (34 mg, 0.06 mmol, 60%) as a white solid. R_f = 0.14 (EtOAc/heptane, 1/1). $[\alpha]_D^{20}$ = −8.8 (c = 1.01, DCM). FTIR (ATR): ν = 3356, 2980, 2254, 1753, 1714 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.79 (s, 1H), 5.87 (d, *J* = 8.8 Hz, 1H), 5.75 (bd, *J* = 5.7 Hz, 1H), 5.45 (t, *J* = 9.3 Hz, 1H), 5.40 (t, *J* = 9.3 Hz, 1H) 5.25 (dd, *J* = 10.0, 9.4 Hz, 1H), 4.96 (bs, 1H), 4.26 (dd, *J* = 12.7, 5.1, 1H), 4.19 (dd, *J* = 12.7, 2.2 Hz, 1H), 4.05−4.01 (ddd, *J*= 10.2, 5.1, 2.1 Hz, 1H), 3.28 (dd, *J* = 15.0, 5.3 Hz, 1H), 3.20 (dd, *J* = 15.0, 5.5 Hz, 1H), 2.09 (s, 3H), 2.08 (s, 3H), 2.04 (s, 3H), 1.9 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.5, 169.8, 169.3, 169.2, 154.3, 142.1, 121.6, 118.0, 86.2, 81.2 75.6, 72.7, 70.8, 68.1, 61.9, 42.5, 29.7, 28.7, 21.2, 21.1, 21.0, 20.7. HRMS (ESI) *m/z* calculated for $C_{24}H_{33}N_5O_{11}Na$ $(M+Na)^+$: 590.2075, found: 590.2080.

Ts-(*S*)-T4M(1-[β-D-Glc(Bn)$_4$])-OMe (61)



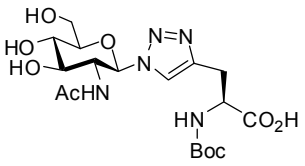
Preparation according to general procedure A for the cycloaddition afforded **61** (57.0 mg, 0.067 mmol, 82%) as a white solid. R_f = 0.34 (EtOAc/heptane, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.68 (d, *J* = 8.2 Hz, 2H), 7.52 (s, 1H), 7.34−7.20 (m, 18H), 7.19−7.15 (m,2H), 6.99−6.96 (m, 2H), 5.56−5.52 (m, 2H), 4.90−4.89 (m, 2H), 4.86 (ABd, *J* = 10.7 Hz, 1H), 4.61 (ABd, *J* = 10.9 Hz, 1H), 4.56 (ABd, *J* = 11.9 Hz, 1H), 4.50 (ABd, *J* = 12.1 Hz, 1H), 4.45 (ABd, *J* = 10.7 Hz, 1H), 4.22 (dt, *J* = 8.4, 5.3 Hz, 1H), 4.03 (ABd, *J* = 10.6 Hz, 1H), 4.00−3.96 (m, 1H), 3.85−3.65 (m, 5H), 3.46 (s, 3H), 3.21 (ABdd, *J* = 15.0, 5.1 Hz, 1H), 3.14 (ABdd, *J* = 15.0, 5.1 Hz, 1H), 2.39 (s, 3H).

Boc-(*S*)-T4M(1-[β-D-GlcNAc(Ac)$_3$])-OH (63)



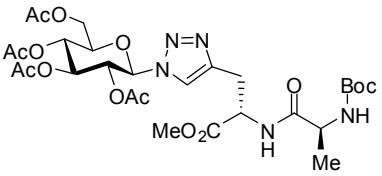
Preparation according to general procedure A for the cycloaddition afforded **63** (1.25 g, 2.14 mmol, 86%) as a white solid. FTIR (ATR): ν = 1744, 1364, 1213 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.75 (s, 1H), 6.48 (br d, *J* = 9.6 Hz, 1H), 5.95 (d, *J* = 10.0 Hz, 1H), 5.62 (d, *J* = 8.0 Hz, 1H), 5.39 (dd, *J* = 10.8, 10.0 Hz, 1H), 5.24 (t, *J* = 9.6 Hz, 1H), 4.71−4.55 (m, 2H), 4.30 (dd, *J* = 12.8, 4.8 Hz, 1H), 4.18−4.10(m, 1H), 4.07−4.01 (m, 1H), 3.34 (dd, *J* = 15.2, 5.2, 1H), 3.23 (dd, *J* = 15.2, 4.8, 1H), 2.08 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 1.80 (s, 3H), 1.45 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.6, 170.7, 170.4, 170.3, 169.1, 155.4, 142.9, 121.7, 86.2, 80.0, 75.1, 72.3, 68.2, 61.9, 53.7, 53.3, 52.6, 28.8, 28.6, 23.0, 21.0, 21.0, 20.9. HRMS (ESI): *m/z* calculated for $C_{24}H_{35}O_{12}N_5Na$ $(M+Na)^+$ 608.21799, found 608.21924.

Boc-(*S*)-T4M(1-[β-D-GlcNAc])-OH (66)



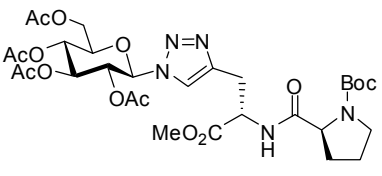
Preparation according to general procedure A for the cycloaddition afforded **66** (22 mg, 0.05 mmol, 53%) as a white solid. FTIR (ATR): ν = 3347, 2971, 2933, 2358, 2340, 2111 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.92 (s, 1H), 5.77 (d, *J* = 9.8 Hz, 1H), 4.44−4.37 (m, 1H), 4.17 (t, *J* = 9.9 Hz, 1H), 3.89 (dd, *J* = 12.3 Hz, 1H), 3.78−3.67 (m, 3H), 3.61−3.43 (m, 2H), 3.22 (dd, *J* = 14.9 Hz, 1H), 3.12 (dd, *J* = 14.9 Hz, 1H), 1.79 (s, 3H), 1.42 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 175.3, 173.5, 157.7, 144.8, 123.0, 88.0, 81.3, 80.6, 75.6, 71.5, 62.4, 56.9, 54.9, 29.1, 28.7, 22.6. LRMS *m/z* calculated for $C_{18}H_{29}N_5O_9$ $(M-H)^-$: 458.2, found: 458.3

Boc-Ala-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-OMe (68)



Preparation according to general procedure A for the cycloaddition afforded **68** (70 mg, 0.11 mmol, 86%) as a white solid. R_f = 0.18 (EtOAc/heptane, 2/1). $[\alpha]_D^{20}$ = −1.3 (c = 0.80, DCM). FTIR (ATR): ν = 3365, 2971, 2358, 2250, 1748, 1511 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.63 (s, 1H), 6.88 (d, *J* = 7.8 Hz, 1H), 5.82 (d, *J* = 9.2 Hz, 1H), 5.42 (t, *J* = 9.5 Hz, 1H), 5.34 (t, *J* = 9.4 Hz, 1H), 5.27−5.17 (m, 1H), 5.23 (dd, *J* = 10.2, 9.2 Hz, 1H), 4.90 (dt, *J* = 8.0, 5.1 Hz, 1H), 4.33 (dd, *J* = 12.7, 5.3 Hz, 1H), 4.25−4.12 (m, 1H), 4.15 (dd, *J* = 12.7, 2.2 Hz, 1H), 4.00 (ddd, *J* = 10.2, 5.1, 2.1 Hz, 1H), 3.76 (s, 3H), 3.34 (dd, *J* = 15.0, 5.1 Hz, 1H), 3.26 (dd, *J* = 15.0, 5.1 Hz, 1H), 2.09 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 1.90 (s, 3H), 1.45 (s, 9H), 1.35 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.4, 170.9, 170.3, 169.6, 169.1, 169.0, 155.1, 143.3, 120.5, 85.9, 80.0, 75.4, 72.6, 70.6, 67.9, 61.7, 52.9, 51.6, 50.3, 28.6, 28.0, 21.0, 20.9, 20.8, 20.4, 18.9. HRMS (ESI) *m/z* calculated for $C_{28}H_{41}O_{14}N_5Na$ $(M+Na)^+$: 694.2548, found: 694.2544.

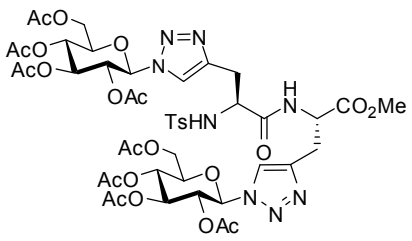
Boc-Pro-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-OMe (70)



Preparation according to general procedure A for the cycloaddition afforded **70** (48 mg, 0.07 mmol, 92%) as a white solid. R_f = 0.14 (EtOAc/heptane, 2/1). $[\alpha]_D^{20}$ = −33.7 (c = 1.03, DCM). FTIR (ATR): ν = 3360, 3140, 2980, 2358, 2336, 2254, 1752, 1679, 1524 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.62 (br

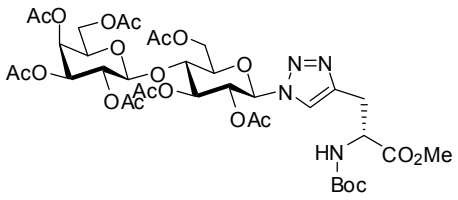
s, 1H), 7.15−6.91 (m, 1H), 5.89−5.74 (m, 1H), 5.44−5.32 (m, 2H), 5.26−5.12 (m, 1H), 4.95−4.76 (m, 1H), 4.34−4.09 (m, 3H), 3.99 (ddd, *J* = 10.0, 4.9, 2.2 Hz, 1H), 3.71 (s, 3H), 3.52−3.34 (m, 2H), 3.30−3.20 (m, 2H), 2.20−1.96 (m, 2H), 2.05 (s, 3H), 2.04 (s, 3H), 2.00 (s, 3H), 1.92−1.75 (m, 2H), 1.85 (s, 3H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.3, 170.9, 170.2, 169.7, 169.2, 168.8, 145.7, 143.5, 120.7, 86.0, 80.5, 75.5, 73.1, 70.7, 68.3, 62.0, 61.2, 52.8, 51.9, 47.3, 28.9, 28.8,24.4, 21.0, 20.9, 20.8, 20.4. HRMS (ESI) *m/z* calculated for $C_{30}H_{43}O_{14}N_5Na$ $(M+Na)^+$: 720.2704, found: 720.2732.

Ts-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-OMe (72)



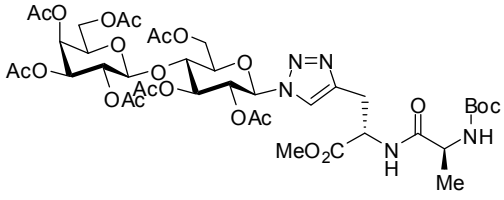
Preparation according to general procedure A for the cycloaddition afforded **72** (57 mg, 0.05 mmol, 55%) as a white solid. R_f 0.17 (EtOAc/heptane, 5/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.57 (s, 1H), 7.69−7.68 (m, 3H), 7.58 (d, *J* = 5.8 Hz, 1H), 7.32 (d, *J* = 8.0 Hz, 2H, 6.24−6.19 (m, 1H), 5.86−5.82 (m, 2H), 5.67−5.63 (m, 2H), 5.50−5.45 (m, 1H), 5.44−5.36 (m, 2H), 5.30−5.26 (m, 1H), 4.79−4.75 (m, 1H), 4.34−4.29 (m, 2H), 4.27−4.22 (m, 1H), 4.20−4.14 (m, 2H), 4.11−4.05 (m, 1H), 4.02 (ddd, *J* = 10.2, 4.5, 1.8 Hz, 1H), 3.85 (s, 3H), 3.61 (ABdd, *J* = 14.7, 4.4 Hz, 1H), 3.53 (ABdd, *J* = 15.6, 2.0 Hz, 1H), 3.30 (ABdd, *J* = 14.7, 4.4 Hz, 1H), 2.78 (ABdd, *J* = 15.4, 6.8 Hz, 1H), 2.44 (s, 3H), 2.12 (s, 3H), 2.09 (s, 3H), 2.09 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.91 (s, 3H), 1.83 (s, 3H), 1.77 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.1, 170.7 170.0 169.5, 169.0, 168.6, 145.5, 143.3, 120.5, 85.8, 80.3, 75.3, 72.9, 70.5, 68.1, 61.8, 52.6, 51.7, 47.1, 28.6, 28.3, 24.2, 20.8, 20.7, 20.6, 20.3.

Boc-(*R*)-T4M(1-[β-D-Lac(Ac)$_7$])-OMe (74)



Preparation according to general procedure A for the cycloaddition afforded **74** (74 mg, 0.08 mmol, 83%) as a white solid. R_f = 0.17 (EtOAc/heptane, 2/1). $[\alpha]_D^{20}$ = −17.8 (c = 1.08, DCM). FTIR (ATR): ν = 3378, 2980, 2366, 2250, 1744 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.50 (s, 1H), 5.75 (d, *J* = 9.0 Hz, 1H), 5.44−5.29 (m, 4H), 5.08 (dd, *J* = 10.4, 7.9 Hz, 1H), 4.94 (dd, *J* = 10.4, 3.3 Hz, 1H), 4.60−4.53 (m, 1H), 4.50 (d, *J* = 7.8 Hz), 4.43 (d, *J* = 11.9 Hz), 4.13−4.03 (m, 4H), 3.94−3.86 (m, 3H), 3.67 (s, 3H), 3.21 (dd, *J* = 14.7, 5.4 Hz, 1H), 3.14 (dd, *J* = 14.7, 4.6 Hz, 1H), 2.11 (s, 3H), 2.06 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.92 (s, 3H), 1.83 (s, 3H), 1.39 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.6, 170.3, 170.1, 170.0, 170.0, 169.3, 169.2, 169.0, 155.3, 143.4, 120.7, 101.3, 85.8, 80.2, 76.3, 76.0, 72.7, 71.2, 71.2, 70.8, 69.4, 67.0, 62.0, 61.2, 53.3, 52.8, 28.9, 28.8, 21.3, 21.2, 21.2, 21.1, 21.1, 21.0, 20.6. HRMS (ESI) *m/z* calculated for $C_{37}H_{53}N_4O_{21}$ $(M+H)^+$: 889.3202, found: 889.3248.

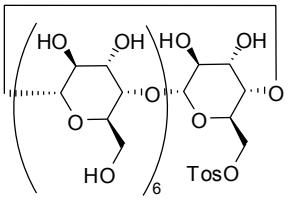
Boc-Ala-(*S*)-T4M(1-[β-D-Lac(Ac)$_7$])-OMe (75)



Preparation according to general procedure A for the cycloaddition afforded **75** (117 mg, 0.12 mmol, 81%) as a white solid. R_f = 0.13 (EtOAc/heptane, 2/1). $[\alpha]_D^{20}$ = −0.5 (c = 1.13, DCM). FTIR (ATR): ν = 3382, 3144, 2980, 2362, 2254, 1752, 1502 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.58 (s, 1H), 6.94 (d,

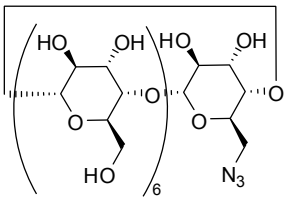
J = 7.8 Hz, 1H), 5.78 (d, *J* = 9.3 Hz, 1H), 5.41−5.25 (m, 4H), 5.14 (dd, *J* = 10.5, 7.8 Hz, 1H), 4.99 (dd, *J* = 10.2, 3.4 Hz, 1H) 4.89 (dt, *J* = 8.0, 5.2 Hz, 1H), 4.55−4.48 (m, 2H), 4.21−4.10 (m, 4H), 3.95−3.40 (m, 3H), 3.75 (s, 3H), 3.32 (dd, *J* = 15.1, 4.9 Hz, 1H), 3.25 (dd, *J* = 15.1, 5.4 Hz, 1H), 2.17 (s, 3H), 2.12 (s, 3H), 2.08 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 1.98 (s, 3H), 1.89 (s, 3H), 1.44 (s, 9H), 1.33 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.4, 170.9, 170.1, 170.0, 169.8, 169.8, 169.2, 169.2, 168.8, 155.1, 143.1, 120.4, 101.1, 85.7, 80.0, 76.2, 75.8, 72.4, 71.0, 71.0, 70.8, 69.2, 66.7, 61.8, 61.0, 52.8, 51.5, 50.3, 28.6, 28.1, 21.1, 21.0, 21.0, 20.9, 20.9, 20.8, 20.5, 18.8. HRMS (ESI): *m/z* calculated for $C_{40}H_{57}O_{22}N_5Na$ $(M+Na)^+$: 982.3393, found: 982.3440.

Mono–6–tosyl–6–deoxy–*β*–cyclodextrin (77)



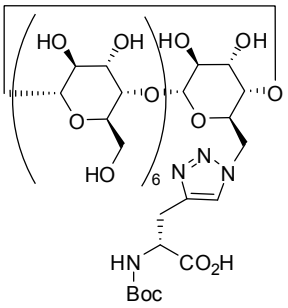
β–Cyclodextrin was dried at 110 °C under high vacuum for 24 h. Subsequently the *β*–cyclodextrin (1.71 g, 1.51 mmol) was dissolved in pyridine (20 mL) and cooled in an ice bath. A solution of tosyl chloride (256 mg, 1.34 mmol) in pyridine (2 mL) was added dropwise to the mixture and stirred for 20 h. The mixture was concentrated *in vacuo,* taken up in slightly acidic water (pH 5) and washed with DCM (2 ×). Next, The water layer was concentrated to yield a white solid. Recrystallization from water yielded the crude mono–6–tosyl–6–deoxy–*β*–cyclodextrin **77** (811 mg, 0.63 mmol). HRMS (ESI) *m/z* calculated for $C_{49}H_{76}O_{37}SNa$ $(M+Na)^+$: 1311.3684, found: 1311.3688.

Mono–6–azido–6–deoxy–*β*–cyclodextrin (78)



The crude mono–6–tosyl–6–deoxy–*β*–cyclodextrin **78** was dissolved in DMF , NaN_3 (39.2 mg, 0.63 mmol) was added and the reaction mixture was stirred for 5 h at 90 °C. Subsequently the mixture was concentrated *in vacuo* and the crude product was purified by several flash column chromatography steps using water/acetronitrile mixtures to afford the mono azide (196 mg, 0.17 mmol, 15% calculated from **76**). HRMS (ESI) *m/z* calculated for $C_{42}H_{69}O_{34}N_3Na$ $(M+Na)^+$: 1182.3660, found: 1182.3683.

Boc-Ala-D-T4M(1-[β-6–deoxy–*β*–cyclodextrin-6-yl])-OMe (79)



A mixture of $Cu(OAc)_2$ (6.8 mg, 0.034 mmol) and sodium ascorbate (14.0 mg, 0.071 mmol) in water (1 mL) was added to a solution of **78** (50.2 mg, 0.04 mmol) and **25** (20.3 mg, 0.09 mmol) in *tert*-butanol (1 mL). The resulting mixture was stirred for 8 h, subsequently the methyl ester was saponified by treatment with NaOH, after which the product was purified by ion exchange chromatography, affording the cyclodextrin–amino acid triazole–linked product **80** (48 mg, 0.03 mmol 81%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.64 (s, 1H) (relevant peaks). HRMS (ESI) *m/z* calculated for $C_{52}H_{84}O_{38}N_4Na$ $(M+Na)^+$: 1395.4661, found: 1395.4720.

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Organic chemistry is the study of carbon compounds.
Biochemistry is the study of carbon compounds that crawl.

-Michael D. Adams

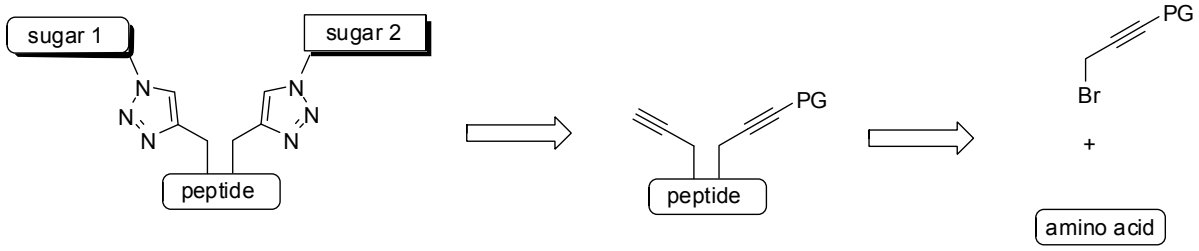
A scientific man ought to have no wishes,
no affections, - a mere heart of stone.

-Charles Darwin

Stepwise Orthogonal Coupling Procedure for the Synthesis of Glycopeptide Mimics

CHAPTER 4

Abstract:
A range of protective groups was examined for application in an orthogonal coupling procedure *en route* to triazole analogues of natural glycopeptides with multiple glycosylated side-chains. Both TMS- and TBDPS-protected propargylglycine derivatives were prepared *via* an alkylation strategy, while subsequent enzymatic resolution afforded the enantiopure TMS-derivative. Subsequently, a variety of pentapeptides, bearing a free as well as a protected (TMS or TBDPS) acetylene side-chain, were prepared *via* solid-phase chemistry. A stepwise [3+2] cycloaddition, deprotection and again [3+2] cycloaddition, either on a resin but also in solution afforded the desired diglycosylated glycopeptides.



4.1 INTRODUCTION

It is believed that in Nature more than 50% of the known glycoproteins are heavily glycosylated with multiple carbohydrate moieties bound to the peptide side-chains,[1] as exemplified by the imaginative glycopeptide **1** (Figure 1). Glycosylation occurs predominantly at the asparagine side-chain, resulting in so-called *N*-glycoproteins, although glycosylation at other sites occurs also frequently, specifically on serine and threonine, giving rise to *O*-glycoproteins.[2] Unfortunately, the synthesis of both *N*- and *O*-glycopeptides is still a tremendous task, partially due to the inherent instability of the glycosidic linkages of the products. We were intrigued, therefore, whether we could extend our research on triazole isosteres of glycoamino acids[3] to triazole-linked analogues (**2**) of *N*-glycoproteins, in particular glycoproteins bearing multiple glycosyl side-chains.

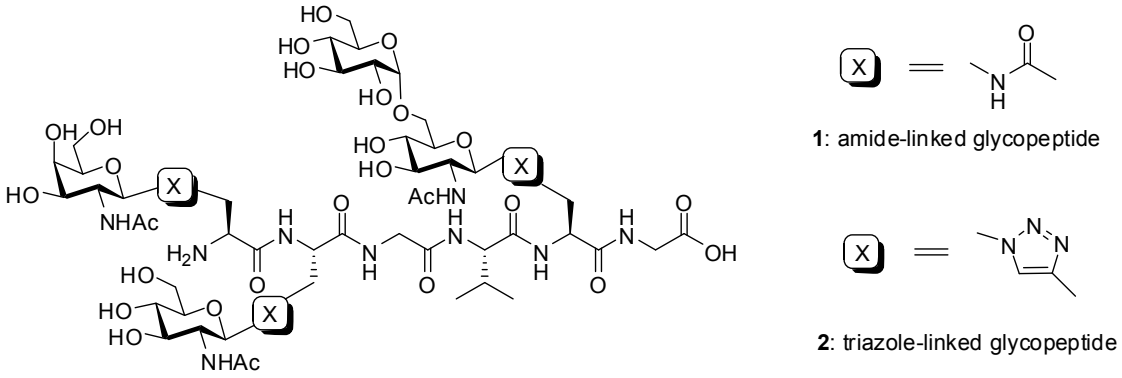


Figure 1. *Amide- (**1**) and triazole- (**2**) linked multi-glycosylated peptide.*

Various strategies can be envisaged to obtain peptides bearing multiple carbohydrates, as illustrated in Figure 2. Thus, the desired peptide with "sugar 1" and "sugar 2" in the side-chain can be prepared in three distinct fashions (Figure 2): (a) by peptide coupling of two separately synthesized triazole-linked glycoamino acids building blocks (route a), (b) by incorporation of an acetylenic amino acid in a peptide already containing one glycoamino acid in the chain (route b), followed by [3+2] cycloaddition, or (c) by synthesis of a peptide containing both a terminal and a protected acetylene, thereby allowing sequential [3+2] cycloaddition, acetylene deprotection, and again [3+2] cycloaddition (route c). Routes a and b are obviously preferable for the preparation of a small number of short, glycosylated

peptides. Although more advanced glycoamino acid building blocks are required, relatively few steps are needed for the peptide synthesis itself. On the other hand, for the assembly of a larger variety of differentially glycosylated glycopeptides, both routes a and b are more laborious due to the requisite investment in glycoamino acid building blocks. The third approach (c) stands out in terms of simplicity to obtain the peptide backbone in large quantity, thereby providing a versatile scaffold for the preparation of a large variety of different triazole-linked glycopeptides *via* sequential incorporation of glycosides. Obviously, the latter approach is especially attractive for the combinatorial preparation of libraries needed for screening purposes.

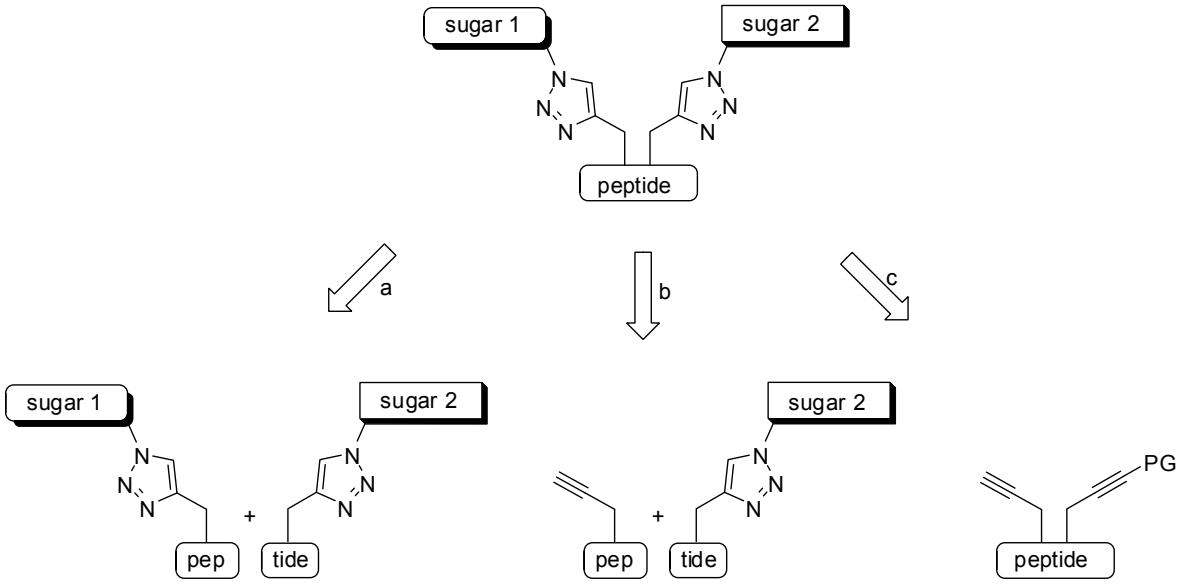


Figure 2. *Different strategies for the preparation of peptides bearing multi-carbohydrate moieties.*

4.2 PROTECTED ACETYLENES

With the objective to prepare a peptide containing a combination of free and protected acetylenes, suitable protected acetylenic amino acids were needed first. An interesting protective group that was initially considered, due to the fact that it can be readily introduced on alkynes in one step, is the dicobalt hexacarbonyl complex **3** (Figure 3). Additionally, we envisioned that straightforward replacement of the acetylene proton by a bromide (**4**) could also serve as protection for the [3+2] cycloaddition, because it is generally accepted that only terminal acetylenes participate in the copper-catalyzed Huisgen cycloaddition. Apart from that, common

acetylene protective groups based on silicon as in compound **5** (*e.g.* $SiMe_3$ (TMS), $SiPh_2{}^tBu$ (TBDPS), etc.) were also considered highly versatile.

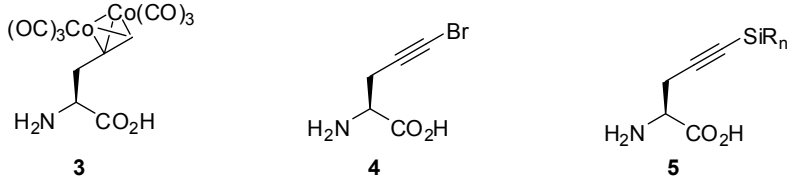
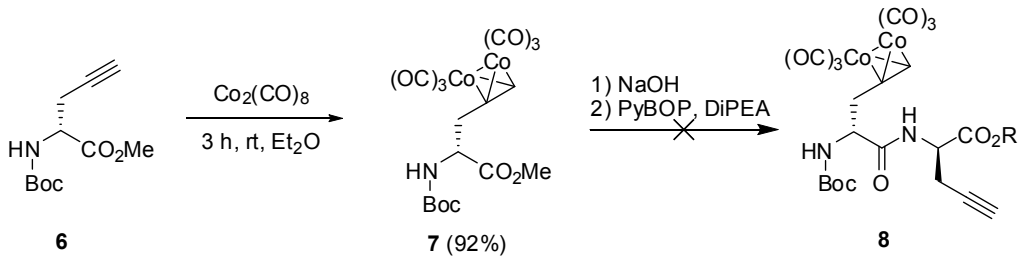


Figure 3. *Different side-chain protected propargylglycine derivatives.*

4.2.1 Cobalt hexacarbonyl complex

The first acetylene protective group that was investigated involved the cobalt hexacarbonyl complex, a remarkably stable bridged structure involving two carbons and two cobalt atoms. In order to determine the applicability of the cobalt hexacarbonyl complex for our purposes, Boc-protected (*R*)-propargylglycine **6** was reacted under typical conditions with dicobalt octacarbonyl in ether, providing the dicobalt hexacarbonyl complex **7** in a high yield (Scheme 1).



Scheme 1. *Formation of the dicobalt hexacarbonyl complex.*

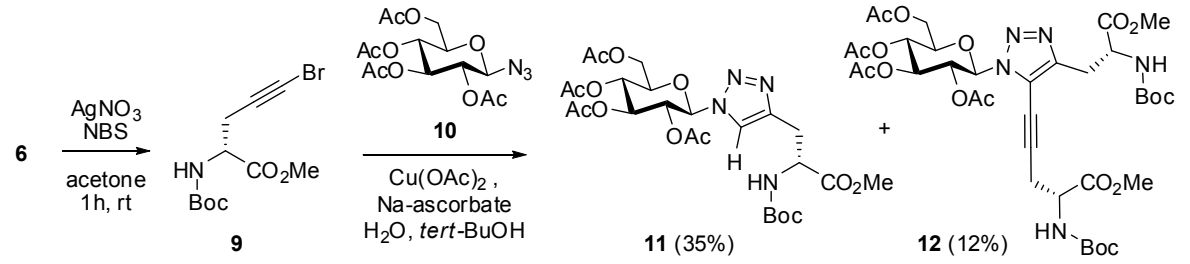
Subsequently, the compatibility of cobalt complex **7** with the typical copper-catalyzed [3+2] cycloaddition conditions was determined. Therefore, cobalt complex **7**, azidosaccharide, $Cu(OAc)_2$ and Na-ascorbate were dissolved in a mixture of *tert*-butyl alcohol and water and stirred for 24 hours at room temperature. As anticipated, no reaction occurred and the cobalt complex could be recovered in high yield, thus paving the way to an orthogonal pathway towards double functionalized peptides.

With the aim of conducting a preliminary test to prepare peptides with multiple functionalities in the side-chains, a dipeptide was required bearing both a free and a "cobalt" protected acetylene. Consequently, methyl ester **7** was saponified and

coupled to D-propargylglycine methyl ester under the action of PyBOP. Much to our surprise, a complex mixture of compounds was formed, either containing or lacking cobalt functionality as could be clearly judged by TLC. Although no detailed analysis of the reaction products was executed, possible side-reactions known to occur in the presence of multiple acetylenes include alkyne-alkyne coupling reactions[4] and cobalt carbonyl-mediated alkyne trimerization.[5] Unfortunately, no major product could be isolated from the complex mixture, although some products were identified that appeared to result from inter- and intramolecular cobalt hexacarbonyl migration from one acetylene to another (not depicted). As a result, the many side-reactions that occurred made the cobalt complex useless for the preparation of multiglycosylated peptides.

4.2.2 Acetylenic bromide

Normally, introduction of bromide on a terminal acetylene will not be considered as a protecting group. However, replacement of the acetylene hydrogen with any other atom was assumed to render it unreactive under the mild conditions for copper-catalyzed [3+2] cycloaddition. To test such an assumption, D-propargylglycine derivative **6** was treated with *N*-bromosuccinimide and a catalytic amount of silver nitrate in acetone[6] to afford bromopropargylglycine **9** (Scheme 2). Unfortunately, a subsequent experiment to determine the compatibility of **9** with the typical conditions for copper-catalyzed cycloaddition proved that the bromide is not suited as a protective group. When bromoacetylene **9** and azidoglucose **10** were subjected to the above mentioned conditions, the 1,4-disubstituted triazole **11** normally formed *via* cycloaddition with the unprotected acetylene was predominantly formed, presumably due to *in situ* debromination followed by the typical [3+2] cycloaddition. Apart from compound **11,** a minor product was formed that appeared to be the remarkable double addition product **12**. The latter findings clearly excluded bromide protection of acetylenes as a coupling strategy, but were the prelude to a range of subsequent experiments for the formation of 1,4,5-trisubstituted triazoles, as described in Chapter 5.



Scheme 2. *Incompatibility of bromoacetylenes with typical conditions for [3+2] cycloaddition.*

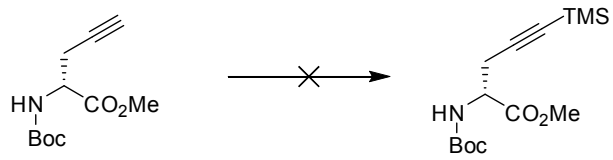
4.2.3 Silyl protective groups

Silicon-based moieties are well-established as protective groups for acetylenes,[7,8] providing excellent stability against a variety of different chemical procedures. Among the silicon substituents, the trimethylsilyl (TMS) is by far the most frequently applied for protection of acetylenes due to the mild basic conditions for silicon removal. Moreover, the fact that some silicon-based protective groups can be orthogonally deprotected was considered convenient for the multiple and selective introduction of a variety of carbohydrates onto peptides. For example, removal of a TMS group normally requires mild base or fluoride ion at ambient temperature, while a *tert*-butyldiphenylsilyl (TBDPS) group often requires a fluoride ion at elevated temperatures to achieve desilylation. Therefore, incorporation of a terminal acetylene, in combination with a TMS and a TBDPS-protected acetylene in a peptide, should allow the stepwise introduction of three (different) carbohydrates *via* intermediate selective deprotection of silyl functions.

4.2.3.1 Acetylene silylation of propargylglycine

Typical conditions for silylation of acetylenes known in literature involve deprotonation of an acetylene with a strong base, followed by addition of an electrophilic silylating reagent such as a silyl chloride. Our first attempts to adapt such a deprotonation-silylation approach to D-propargylglycine **6** (Table 1) involved treatment of **6** with a variety of strong bases, *e.g.* EtMgBr (entry 1), NaHMDS (entry 2) or ^{n}BuLi (entry 3) followed by the addition of chlorotrimethylsilane. Unfortunately, no silylation occurred, but instead partial saponification of the methyl ester was detected in all cases (5-15%). Other methods, involving a Lewis acid to promote silylation[9] (entry 4) or a mild base[10] (entry 5) also failed to provide the desired product **13**.

Table 1. *Direct silylation of Boc-protected propargylglycine.*

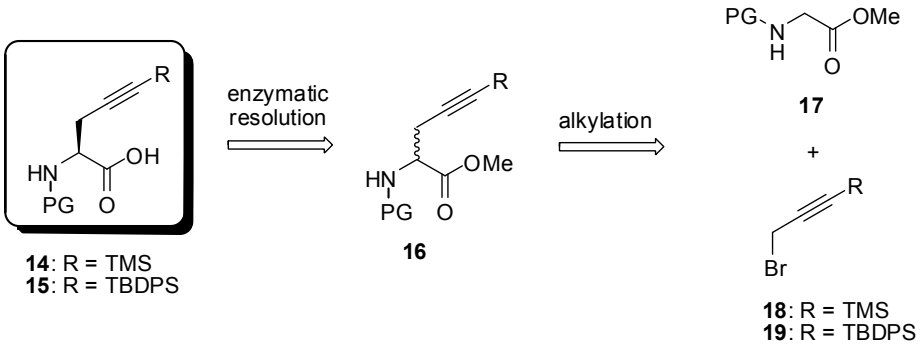


6 **13**

entry	TMS-source	base/catalyst	solvent	result
1	TMSCl	EtMgBr	THF	partial hydrolysis of the ester
2	TMSCl	NaHMDS	THF	partial hydrolysis of the ester
3	TMSCl	^{n}BuLi	THF	partial hydrolysis of the ester
4	TMSCl	$Zn(OTf)_2$ / Et_3N	DCM	no reaction
5	TMSCl	$CsOH \cdot H_2O$	THF:DMSO	hydrolysis of the ester

4.2.3.2 α-Alkylation of glycine

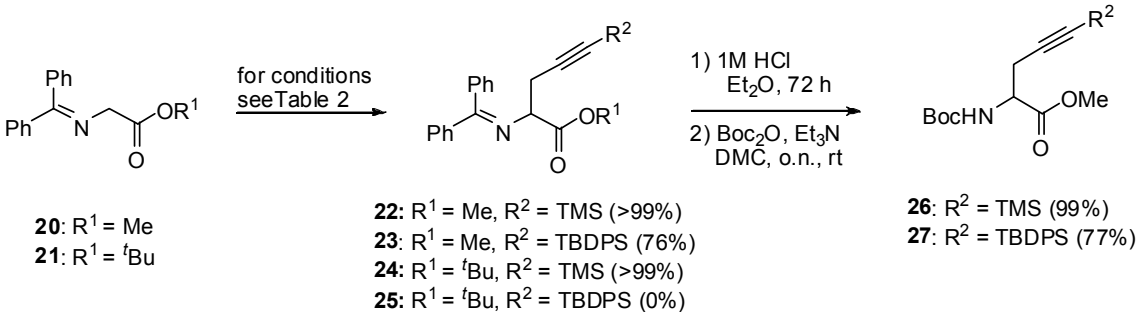
Having found that direct alkylation of enantiopure propargylglycine was not successful, attempts were made to prepare the desired silyl-protected compounds **14** and **15** *via* α-alkylation of glycine derivative **17** with protected propargyl bromides **18**[11] and **19**[12] (Scheme 3). Obviously, such a route provides the intermediate product **16** as a racemic compound. Although asymmetric alkylations[13] are known for similar compounds, a racemic synthesis of **16** followed by enzymatic resolution was considered as easier, economically more feasible for larger quantities,[14] and a better guarantee for a high enantiomeric excess.



Scheme 3. *Retrosynthesis of enantiomerically pure protected propargylglycine derivatives.*

Preparation of TMS-protected propargylglycine was achieved by deprotonation of the diphenylketimine protected esters **20** and **21** with a strong base (KOH) at −40 °C

(Scheme 4 / Table 2). Subsequent addition of TMS-propargyl bromide[11] afforded the alkylated methyl ester **22** and *tert*-butyl ester **24** in excellent yields (Table 2, entry 1 and 2).



20: R^1 = Me
21: R^1 = ^{t}Bu

22: R^1 = Me, R^2 = TMS (>99%)
23: R^1 = Me, R^2 = TBDPS (76%)
24: R^1 = ^{t}Bu, R^2 = TMS (>99%)
25: R^1 = ^{t}Bu, R^2 = TBDPS (0%)

26: R^2 = TMS (99%)
27: R^2 = TBDPS (77%)

Scheme 4. *Preparation of silyl-protected racemic propargylglycines **26** and **27**.*

Alkylation of the ^{t}Bu-ester **21** under identical conditions with TBDPS-protected propargyl bromide[12] as electrophile (entry 4), however, completely failed to afford the desired product **25**. Fortunately, the synthesis of the TBDPS-protected propargylglycine derivate could be achieved upon subjection of methyl ester **20** to LDA,[15] followed by alkylation to afford the desired product **23** in 76% yield (entry 5).

Table 2. *Alkylation of **20** and **21** with protected propargyl bromide derivatives.*

Method A:
TBAB 10 mol%, KOH 50% aq.
25 or **26** 5 equiv
toluene: THF 7:3, 8h, -40 °C

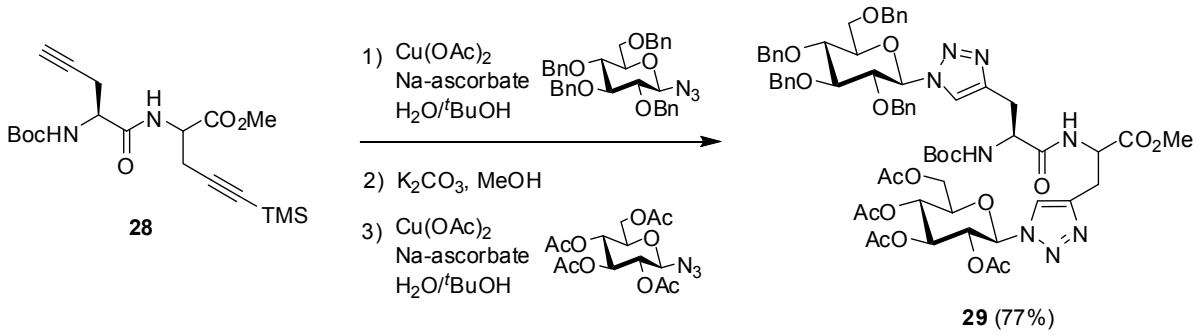
Method B:
1) LDA 1.2 equiv
2) alkylation agent 2 equiv
HMPA 1.2 equiv, THF, 4h, r.t.

entry	R^1	R^2	method	product	yield
1	Me	TMS	A	**22**	>99%
2	^{t}Bu	TMS	A	**24**	>99%
3	Me	TMS	B	**22**	28%
4	^{t}Bu	TBDPS	A	**25**	no reaction
5	Me	TBDPS	B	**23**	76%

Subsequent treatment of the methyl esters **22** and **23** with a 1 M HCl solution effected the hydrolysis of the *N*-diphenylketimine which was followed by Boc–protection of the resulting free amine to afford the Boc-protected TMS- and TBDPS- protected propargylglycine methyl esters **26** and **27** in 99% and 77% yield, respectively.

4.2.3.3 Proof-of-concept of stepwise [3+2] cycloaddition

Having the racemic silyl-protected propargylglycines **26** and **27** at hand, the stage was set to verify the concept of stepwise cycloaddition onto peptides containing differentially protected side-chains. Therefore the diacetylenic dipeptide **28** was prepared by condensing the (racemic) TMS-protected propargylglycine methyl ester **26** with commercially available Boc-(*S*)-propargylglycine *via* standard peptide coupling chemistry.



Scheme 5. *Preparation of a differentially glycosylated triazole-linked glycopeptide.*

The potential of a stepwise introduction of carbohydrates *via* [3+2] cycloaddition was now investigated by first cycloaddition of the dipeptide **28** with tetra-*O*-benzyl azidoglucoside under the typical $Cu(OAc)_2$/Na-ascorbate conditions. As anticipated, a smooth and clear introduction of a single carbohydrate moiety was observed, leaving the trimethylsilyl function fully unaffected. Subsequent desilylation with K_2CO_3 in methanol proceeded uneventfully, followed by another [3+2] cycloaddition, this time involving tetra-*O*-acetylazidoglucose, to afford the triazole-linked diglycosylated peptide **29** in an excellent total yield of 77% for the three steps (Scheme **5**).

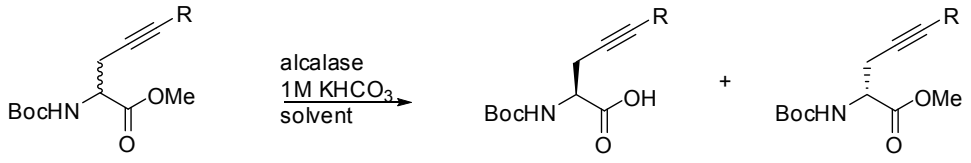
4.2.3.4 Enzymatic resolution of silyl-protected propargylglycine

With the proof-of-concept for stepwise introduction of azides by [3+2] cycloaddition firmly established, enzymatic resolution of the TMS- and TBDPS-protected substrates **26** and **27** became requisite for the preparation of diastereomerically pure peptides. The industrially available *alcalase,* an enzyme mixture produced by *Bacillus licheniformis* containing subtilisin Carlsberg as the major enzyme component, seemed

perfectly suitable[16] for such a purpose, since *alcalase* is known for its high activity and stability in alcoholic solvents.[17]

The first enzymatic resolution of TMS-protected propargylglycine **26** was attempted by suspension of **26** in a mixture of *tert*-amyl alcohol and aqueous $KHCO_3$ (Table 3). Next, a stoichiometric equivalent of *alcalase* solution (a mass equal to the amount of substrate) was added and the resulting mixture was vigorously stirred at 37 °C. After 25 hours, HPLC analysis indicated a yield of 37% of the amino acid (entry 1), which luckily could be increased to 50%, the maximum yield for a classical resolution, upon addition of eight mass-equivalents of *alcalase* (entry 2). Subsequent isolation afforded the (*S*)-amino acid **28** in a yield of 48% with an excellent enantiomeric excess of 98%.

Table 3. *Enzymatic resolution of silyl-protected propargylglycines* **26** *and* **27**.



26: R= TMS
27: R= TBDPS

28: R= TMS
29: R= TBDPS

30: R= TMS
31: R= TBDPS

entry	R	solvent	*alcalase* (mass equiv)	time	conversion to acid	*e.e.* of acid
1	TMS	^{t}amylOH	1	25 h	37%	–
2	TMS	^{t}amylOH	8	24 h	50% (48%)[a]	98%
3	TBDPS	^{t}amylOH	1 – 8	22 h	0%	–
4	TBDPS	^{t}amylOH/DMF (3:1)	8	24 h	23%	–
5	TBDPS	^{t}BuOH/ DMF (3:1)	8	45 h	43% - 50%	3% - 40%

[a]Isolated yield.

Moreover, the reaction profile (Figure 4) revealed the high specificity of the *alcalase*, since prolonged stirring (24-48 h) did not drive the conversion above the theoretical yield of 50% for enantioselective hydrolysis. Contrary to the TMS-protected substrate, subjection of TBDPS-protected propargylglycine **27** to identical conditions failed to show any conversion to the desired amino acid **29** (entry 3), probably due to the poor solubility of **27** in *tert*-amyl alcohol.

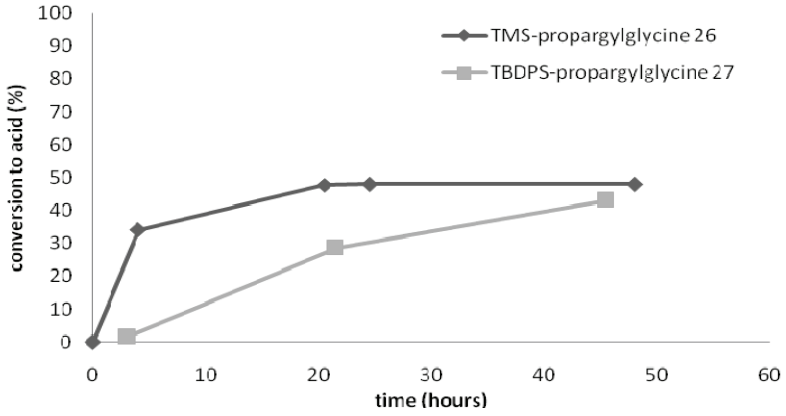


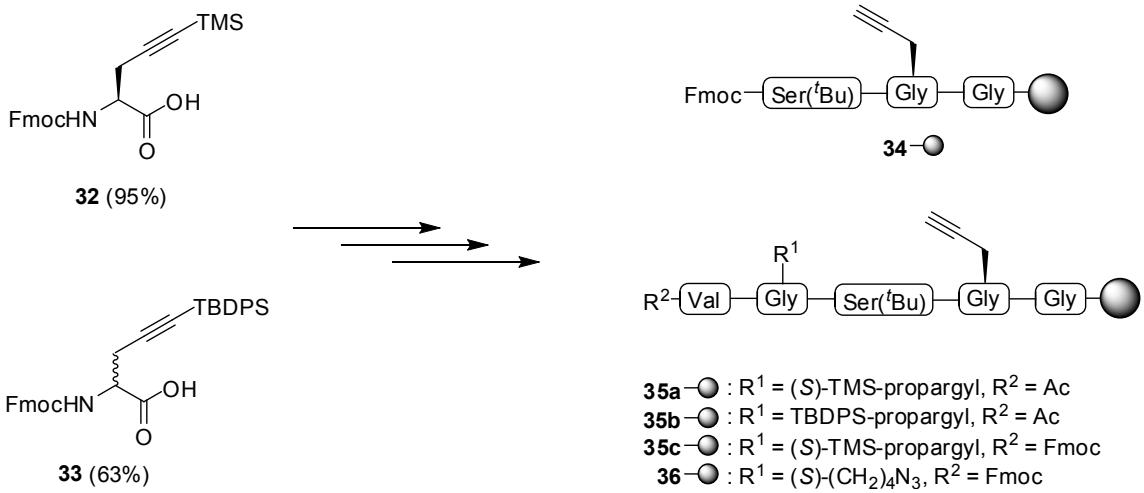
Figure 4. *Enzymatic resolution of* **26** *and* **27**.

In order to enhance the solubility of TBDPS-protected propargylglycine **27**, resolution was attempted in a mixture of DMF, *tert*-amyl alcohol and aqueous $KHCO_3$. After 24 hours, HPLC analysis indicated that indeed conversion was taking place, but only to an extent of 23%. Gratifyingly, substitution of ^{t}amylOH by ^{t}BuOH led to significantly increased conversion of 43-50% of the desired free acid. Nevertheless, it was disappointing to find that the reaction seemed to proceed with a large fluctuation in enantioselectivity, with ee varying from 3 to 40% for reasons unclear to us. Therefore, it was decided to temporarily continue with the racemic TBDPS-protected propargylglycine for incorporation in peptides and to postpone further optimization of the enzymatic resolution to a later time.

4.3 ORTHOGONAL COUPLING ONTO PEPTIDES

4.3.1 Peptide synthesis

The most straightforward and reliable approach for the preparation of a given peptide is by Fmoc-based solid phase peptide chemistry. For this purpose, a trityl resin was selected to ensure that the final protected peptides could be smoothly cleaved from the resin, without removal of the silyl protective groups. As the solid phase approach demands Fmoc groups, the Boc-groups of TMS- (**28**) and TBDPS- (obtained by base saponification of **27**) containing amino acids were removed (TFA, DCM), followed by Fmoc-protection with FmocOSu, to afford the amino acids **32** and **33**, respectively (Scheme 6).



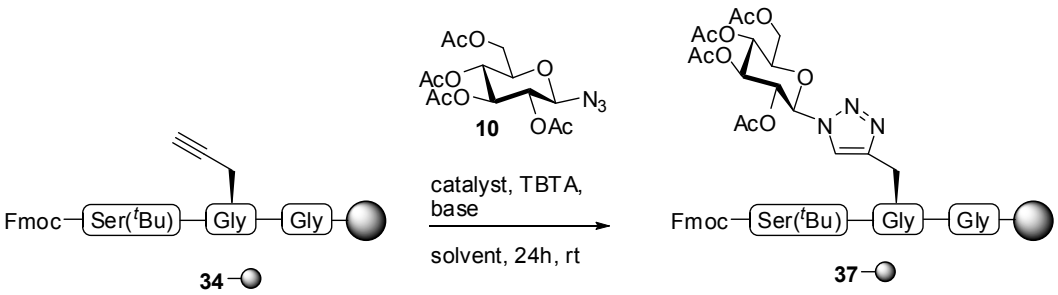
Scheme 6. *Preparation of the peptides.*

Subsequently, peptides **34** and **35a-c** were prepared by standard Fmoc-based peptide chemistry, involving HOBt/DIPCDI-mediated coupling on a *C*-terminal glycine followed by piperidine-induced Fmoc deprotection. The resin-bound tripeptide **34** was prepared to determine the optimal conditions for on-resin [3+2] cycloaddition. Furthermore, an azido-containing peptide **36** including a lysine derivative[18] was synthesized applying the same technique (Scheme 6).

4.3.2 Optimization of on resin [3+2] cycloaddition

Resin-bound tripeptide **34** and tetraacetylated 1-azidoglucose **10** were subjected to various copper-catalyzed [3+2] cycloaddition conditions (Table 4). In order to monitor progression of the reaction, small portions of the peptide were washed, dried, cleaved from the resin and examined with LCQ-MS. Reactions conducted in acetonitrile with Et_3N or 2,6-lutidine/DiPEA as base resulted in the formation of the product although significant amounts of starting material remained after 24 hours (entries 1 and 2). The conversion improved upon utilizing DMF as solvent and a 2,6-lutidine/DiPEA base combination, but again small amounts of starting material were still present after 24 hours (entry 3). Much to our satisfaction, treatment of the peptide with CuI in DMF in the presence of Et_3N or DiPEA led to full conversion to the desired triazole product (entry 4). For comparison, conditions described by Kirshenbaum *et al.*[19] were also attempted (entry 5), but a more sluggish reaction was observed, producing several side-products.

Table 4. *Optimization studies of on-resin [3+2] cycloaddition.*



entry	solvent	catalyst	base	result[a]
1	MeCN	CuI	Et_3N	+/−
2	MeCN	CuI	2,6-lutidine/ DiPEA	+/−
3	DMF	CuI	2,6-lutidine/ DiPEA	+/−
4	DMF	CuI	Et_3N or DiPEA	+
5	DMF: pyridine 7:3	CuI	DiPEA	+[b]

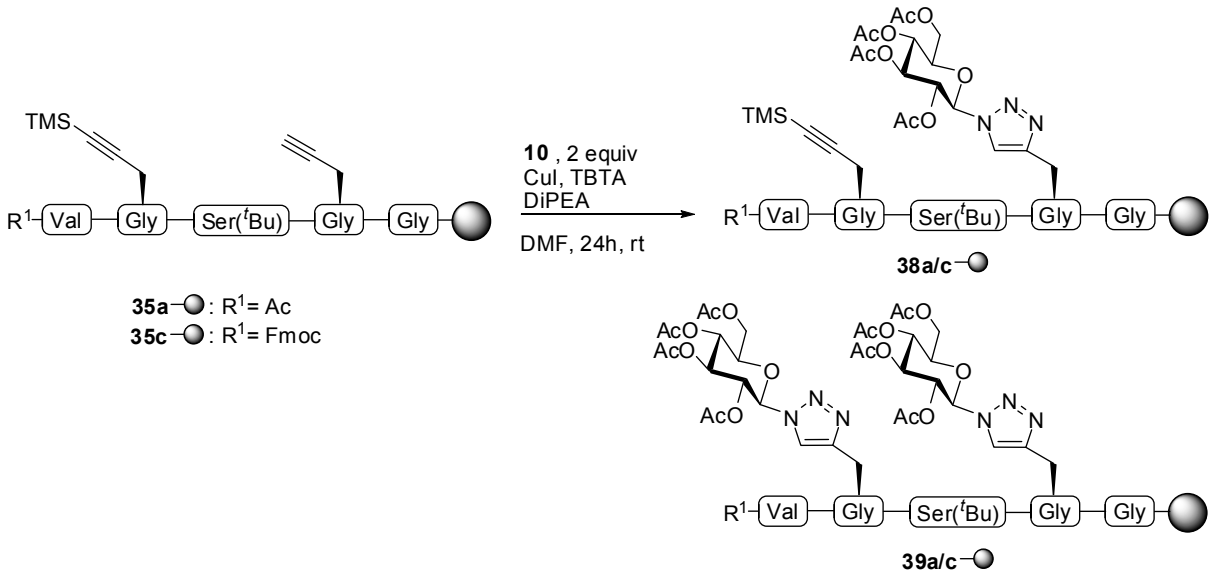
[a] + = Only mass of product found, +/− = Mass of product and s.m. found, − = Only mass of s.m. found [b] Formation of byproduct was evidenced.

4.3.3 Orthogonal couplings on resin

With suitable conditions for [3+2] cycloaddition clearly established, a synthetic route towards multiple glycosylated peptides was investigated. To this end, resin bound pentapeptide **35c** was subjected to the conditions derived in the optimization studies (Table 5, entry 1). Surprisingly, a negligible amount of the desired mono-glycosylated product **38** was formed, but instead a second [3+2] cycloaddition had occurred on the presumed protected TMS-propargylglycine, leading to the diglycosylated product **39a**.

Apparently, *in situ* desilylation had taken place under the conditions employed. At first, complexation of copper(I) to TBTA was believed responsible for the production of an iodide source that is sufficiently nucleophilic to desilylate the acetylene. However, omission of TBTA (entry 2) led to complete absence of product formation. Similar observations were made with Fmoc-protected pentapeptide **35c**, leading to a mixture of **38c** and **39c**. Application of a different copper source (entry 5) or a single equivalent of azidoglucose (entry 4) did not improve matters.

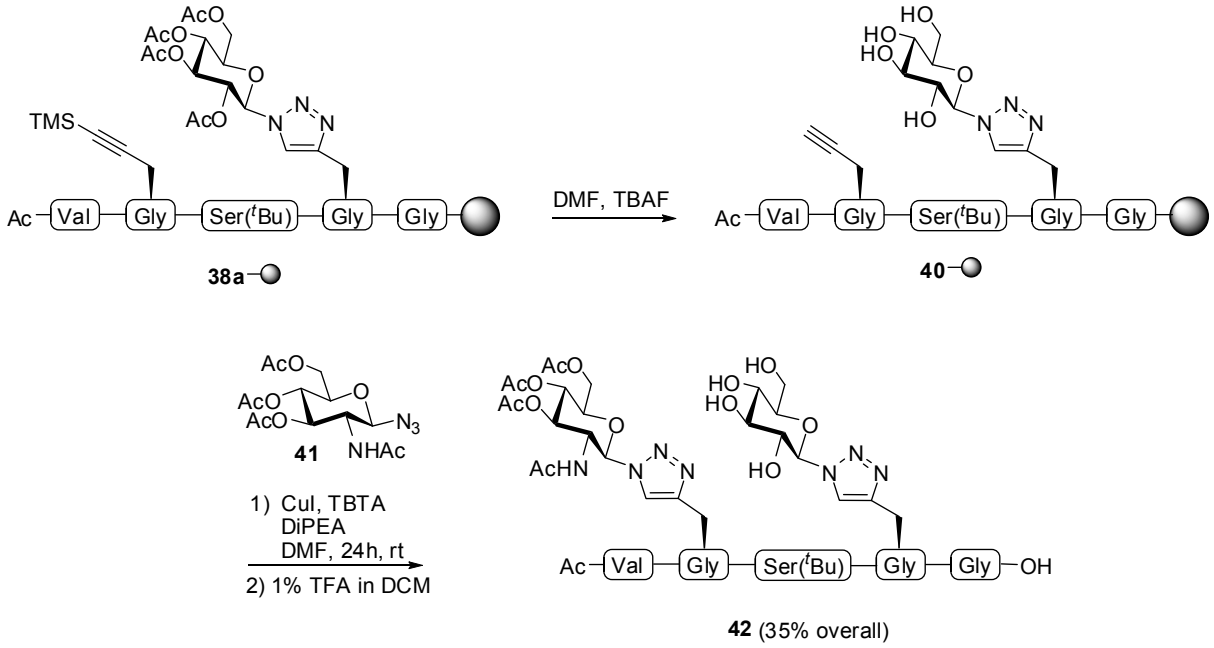
Table 5. *First resin bound [3+2]-cycloaddition.*



entry	R^1	solvent	ligand	product	result[a]
1	Ac	DMF	TBTA	**39a**	**+**
2	Ac	DMF	–	–	–[b]
3	Fmoc	DMF	TBTA	**38c + 39c**	+
4	Fmoc	DMF	TBTA	**38c + 39c**	+[d]
5	Fmoc	DMF	TBTA	–	–[c]
6	Fmoc	MeCN	TBTA	–	–
7	Fmoc	THF	TBTA	**38c**	**+**
8	Ac	THF	TBTA	**38a**	**+**

[a]+ = Only mass of product(s) found, – = Only mass of s.m. found. [b]No TBTA added to reaction mixture. [c]$Cu(MeCN)_4PF_6$ used as catalyst. [d]1 Equiv of. azide added to reaction mixture

In contrast, when the reaction was performed in THF instead of DMF, a dramatic improvement in selectivity took place, leading to the exclusive formation of the desired monoglycosylated products **38c** and **38a,** entries 7 and 8, respectively. A possible explanation for the desilylation encountered in DMF may be found in the increased nucleophilicity of the iodide in DMF, possibly in combination with π-interaction between the triazole-bound copper ion and TMS-acetylene in a diacetylene copper complex[20] formed during the [3+2] cycloaddition. This was substantiated by the fact that control experiments with only TMS-propargylglycine, CuI, Et_3N and DMF did not lead to desilylation.



Scheme 7. *Acetylene deprotection, [3+2] cycloaddition and subsequent cleavage from the resin to* ***42****.*

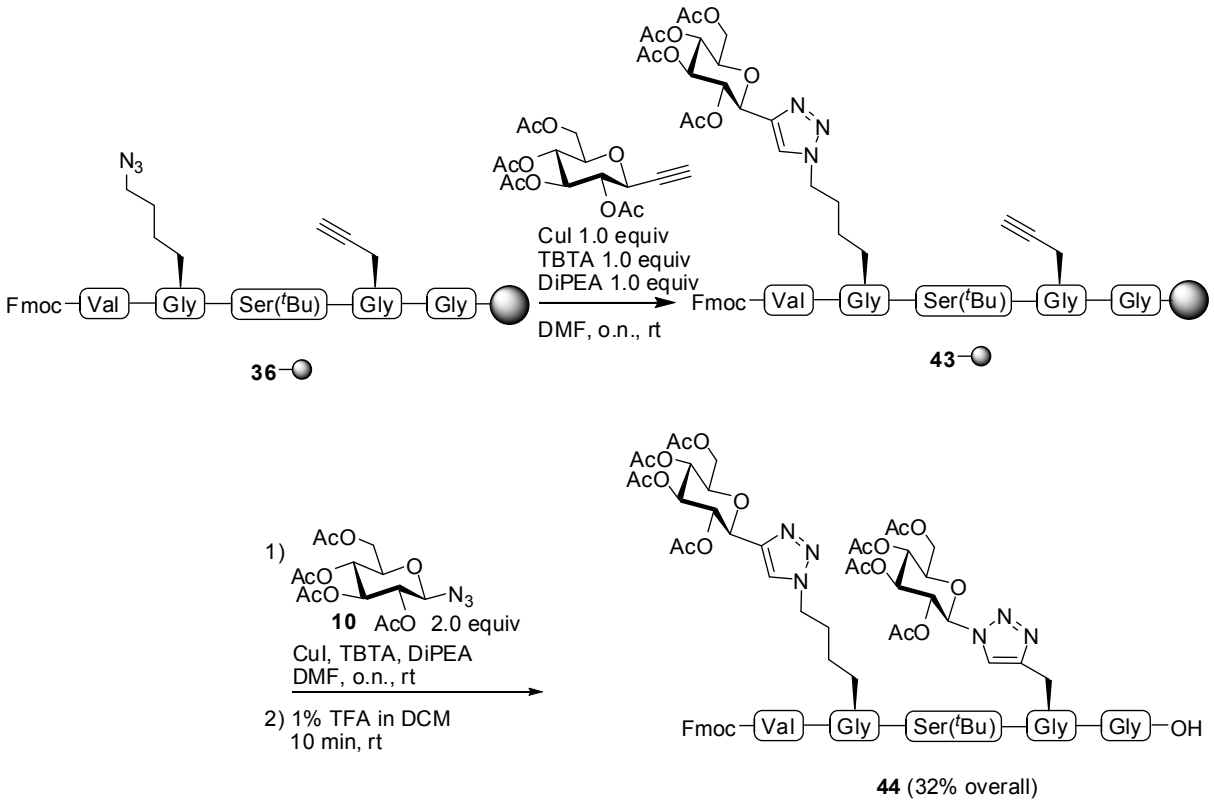
Having successfully introduced a single glycoside onto the monoprotected dipropargylpeptide, deprotection of the TMS-acetylene of the resin-bound monoglycosylated pentapeptide **38a** was investigated under a variety of conditions. However, none of the known solution phase conditions for desilylation,[7] *e.g.* K_2CO_3/MeOH, NaOMe, NaH and NaOH led to deprotection of the acetylene. Nevertheless, finally it was found that treatment of **38a** with TBAF (1 M in THF) led to smooth desilylation along with saponification of acetyl functions, giving rise to resin–bound peptide **40** (Scheme 7). Unfortunately, TMS deprotection with TBAF to some extent also induced cleavage from the resin. Nevertheless, preparation of the final diglycosylated resin bound peptide **42** was achieved by [3+2] cycloaddition of the TMS-deprotected intermediate **40** with tetra-*O*-acetyl-1-azidoglucosamine **41** in DMF. Subsequent cleavage from the resin afforded the diglycosylated peptide **42** in a promising overall yield of 35% (Scheme 7).

Finally, the same sequence was followed for the TBDPS-protected peptide **35b**. We were pleased to find that the more robust TBDPS-group was fully unaffected by conditions of DMF, CuI and Et_3N, and the pentapeptide **35b** smoothly afforded the monoglycosylated derivative (not depicted). Subsequently, deprotection of the TBDPS-group with concomitant deacetylation to **40** could be achieved upon subjection to TBAF at 40 °C for 6 hours, although also in this case partial peptide

cleavage from the resin was observed. Other methods for the deprotection of the TBDPS group, *e.g.* KF or HF-pyridine gave no desilylation or led to cleavage from the resin, respectively. Naturally, subsequent cycloaddition and cleavage from the resin led to the racemic derivative of **42**.

4.3.3.1 [3+2] Cycloadditions onto a pentapeptide bearing an azidobutylglycine and propargylglycine moiety

Besides the TMS- and TBDPS-protected pentapeptides, an azido-containing amino acid derived from lysine was also incorporated in pentapeptide **36**. The latter peptide was designed for the orthogonal introduction of different carbohydrate moieties *via* [3+2] cycloaddition. Although the peptide bears both an azide and an acetylene function, intramolecular cycloaddition is not likely based on earlier findings in our laboratory.[21]



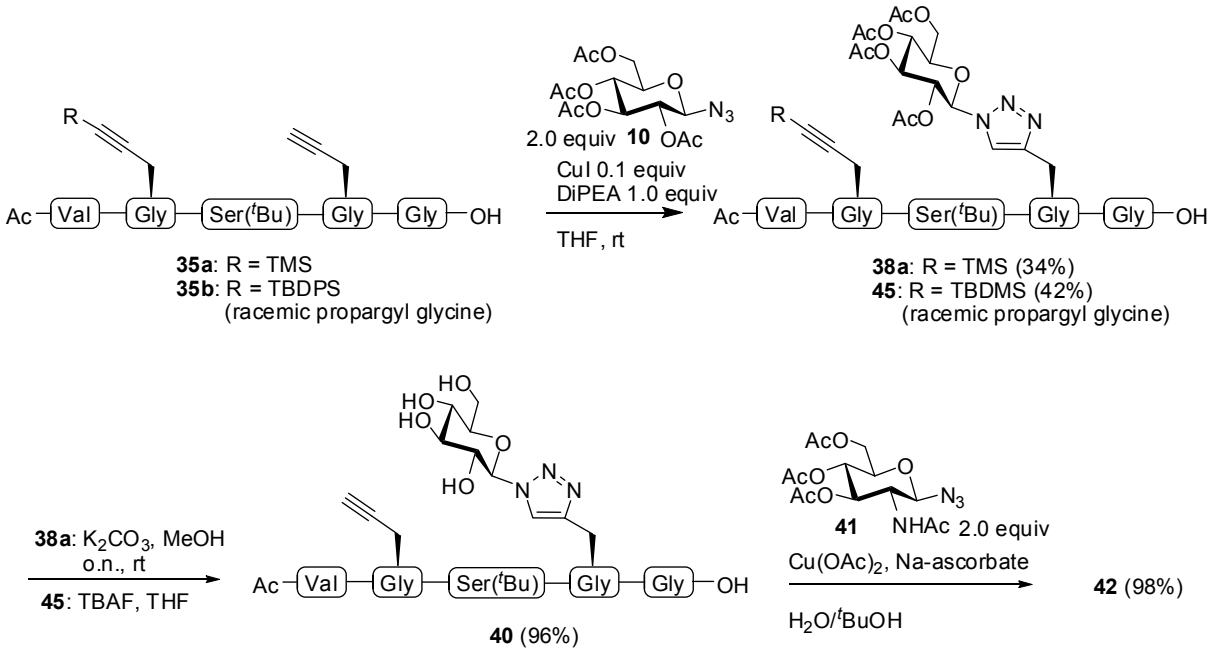
Scheme 8. *Resin bound strategy for the synthesis of a diglycosylated peptide.*

We were gratified to find that introduction of an acetylene-containing glucose derivative to the azidolysine moiety under the optimized conditions proceeded smoothly and selectively, providing resin-bound peptide **43** after 24 hours

(Scheme 8). The resin was washed and without the need for an intermediate deprotection a second [3+2] cycloaddition was performed with azidoglucose derivative **11** under the same conditions providing the diglycosylated peptide **44** in a 32% overall yield after cleavage from the resin (1% TFA in DCM).

4.3.3.2 Orthogonal couplings in solutions

Although solid phase procedures are generally preferred over solution-based protocols for the preparation of peptides (*e.g.* with regard to purification), the difficulties encountered during cycloaddition and desilylation on the solid support stimulated us to investigate whether solution synthesis of the same peptides might be beneficial. Thus, pentapeptides **35a** and **35b** were cleaved from the resin and dissolved in THF before addition of azidoglucose **11**, CuI and DiPEA. The desired triazole adducts **38a** and **45** were formed without incidence and isolated in 34 and 42%, respectively (Scheme 9).



Scheme 9. *Solution phase procedure for the preparation of a diglycosylated peptide.*

Next, removal of the TMS-group from **38a** and simultaneous saponification of acetyls was achieved under the action of K_2CO_3 in MeOH, providing substrate **40** in 96%. For compound **45**, complete desilylation of the TBDPS group was evidenced by TLC and LCQ-MS upon subjection to 1 M TBAF in THF. Finally, pentapeptide **40** was

dissolved in a mixture of *tert*-butyl alcohol and water, due to insolubility in THF, in order to induce the second copper-catalyzed [3+2] cycloaddition with $Cu(OAc)_2$ and Na-ascorbate as described in Chapter 3. It was highly rewarding to find that the latter cycloaddition resulted in a near quantitative yield of the diglycosylated peptide **42**.

4.4 CONCLUSIONS

Stepwise introduction of carbohydrates onto an orthogonally protected peptide was successfully achieved. While cobalt hexacarbonyl and bromide protection proved unsuitable as protection of acetylene function, TMS and TBDPS-protected propargylglycine served the desired goal well. To this end, both silylated propargylglycines were prepared *via* alkylation of a glycine derivative, followed by enzymatic resolution under the action of *alcalase*. Enantiomerically pure L-TMS-protected propargylglycine was obtained in excellent yield, but the TBDPS-counterpart led to variable yields and stereoselectivities appeared hard to reproduce. Both the racemic TBDPS-protected as well as the enantiopure TMS-protected propargylglycine derivative were incorporated in a pentapeptide for stepwise [3+2] cycloaddition, deprotection and again [3+2] cycloaddition, both on resin and in solution. A minor disadvantage of the on resin approach involved partial cleavage of the peptide from the resin during desilylation, while the solution approach was slightly hindered by difficulties encountered during purification, leading to a decrease in total yield. Nevertheless, both the solid phase strategy as well as reactions in solution led to clean double introduction of carbohydrates onto the pentapeptide with a high overall yield of approximately 35%.

4.5 ACKNOWLEDGMENT

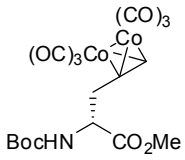
(Rik) T. C. Cleophas is gratefully acknowledged for his large contribution to this chapter. Dr. P. J. L. M. Quaedflieg (DSM Pharmaceutical Products, Geleen, The Netherlands) is acknowledged for the helpful discussions and advice on the enzymatic reactions.

4.6 EXPERIMENTAL

General information. For general experimental details, see Section 2.7

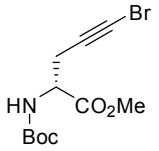
Nomenclature of the peptides. For information on the nomenclature, see Section 3.6

Methyl (2*R*)–2–[(*tert*–butoxycarbonyl)amino]–4–pentynoate dicobalthexacarbonyl complex (7).



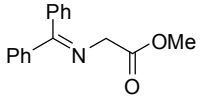
To a solution of (*R*)-Boc-propargylglycine methyl ester (80 mg, 0.35 mmol) in ether (2 mL) was added dicobalt octacarbonyl (128 mg, 0.37 mmol). The resulting mixture was stirred for 4 hours, evaporation of the solvent *in vacuo* and purification by flash column chromatography afforded **7** (167 mg, 0.33 mmol, 92%) as a thick red oil. R_f = 0.34 (EtOAc/heptane, 1/3). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.29 (d, *J* = 7.4 Hz, 1H), 4.48−4.35 (m, 1H), 3.71 (s, 3H), 2.76−2.56 (m, 2H), 1.98 (t, *J* = 2.6 Hz, 1H), 1.38 (s, 9H).

(2*R*)-5-Bromo-2-*tert*-butoxycarbonylamino-4-pentynoic acid methyl ester (9)



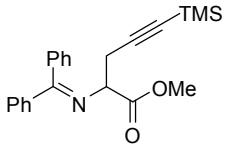
To a solution of (*R*)-Boc-propargylglycine methyl ester (98 mg, 0.43 mmol) in acetone (10 mL) was added *N*-bromosuccinimide (84 mg, 0.47 mmol) and a catalytic amount of $AgNO_3$ (5.0 mg, 0.03 mmol). The mixture was stirred for 1 h, water was added and the product was extracted with pentane, purification by flash column chromatography afforded **9** (91 mg, 0.30 mmol, 69%). R_f = 0.64 (EtOAc/heptane, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.31 (br d, *J* = 7.2 Hz, 1H), 4.49−4.44 (m, 1H), 3.79 (s, 3H), 2.84−2.69 (m, 2H), 1.46 (s, 9H). Spectral data are in accordance with literature.[22]

***N*-(Diphenylmethylene)glycine methyl ester (21)**



A solution of benzophenone imine (1.0 g, 5.52 mmol), glycine methyl ester hydrochloride (0.697 g, 5.52 mol) in DCM (20 mL) was stirred for 24 h at room temperature, filtrated and the organic solvent was evaporated *in vacuo*. The crude product was redissolved in Et_2O (10 mL), filtrated, washed with H_2O, and dried ($MgSO_4$). Subsequent evaporation of the solvent *in vacuo* followed by recrystallization in ether/hexane afforded **21** (1.12 g, 4.43 mmol, 80%). R*f* = 0.35 (EtOAc/heptane, 1/3). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.77−7.65 (m,2H), 7.49−7.32 (m, 6H), 7.19−7.17 (m, 2H), 4.22 (s, 2H), 3.75 (s, 3H). Spectral data are in accordance with literature.[23]

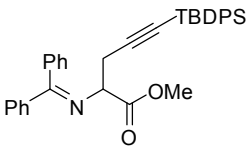
***N*-(Diphenylmethylene)trimethylsilanepropargylglycine methyl ester (22)**



To a mixture of *N*-(diphenylmethylene)glycine methyl ester (**20**) (254 mg, 1.0 mmol), tetrabutyl ammonium bromide (1 equiv) and TMS-propargyl bromide (**18**, 950 mg, 5.0 mmol) in toluene/THF (5 mL, 7:3) was added KOH (1.5 mL, 50% aqueous) at −40 ˚C. The resulting mixture was stirred for 8 hours at −40˚C, water was added and the product was extracted with Et_2O (2 ×). The combined organic layers were washed with saturated aqueous NaCl, dried (Na_2SO_4) and evaporated *in vacuo*. The product was purified by flash column chromatography affording **22** (362 mg, 1.0 mmol, >99%) as a white solid. R*f* = 0.6

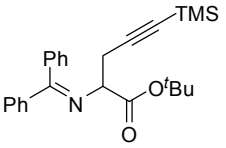
(EtOAc/heptane, 1/2). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.65−7.63 (m, 2H), 7.46−7.28 (m, 6H), 7.19−7.16 (m, 2H), 4.31 (dd, *J* = 7.6, 6.2 Hz, 1H), 3.72 (s, 3H), 2.97−2.78 (m, 2H), 0.11 (d, *J* = 15.1 Hz, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.0, 171.3, 139.8, 136.2, 132.5, 130.5, 130.2, 129.2, 128.9, 128.5, 128.4, 128.1, 103.6, 86.8, 64.8, 52.5, 25.0, 0.1. HRMS (ESI) *m/z* calculated for $C_{22}H_{26}NO_2Si$ $(M+H)^+$: 364.1733, found: 364.1731.

N-(Diphenylmethylene)-(3-*tert*-butyldiphenylsilylpropargyl)glycine methyl ester (23)



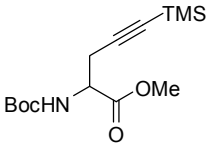
To a solution of *i*-Pr_2NH (1.86 mL, 13.2 mmol), in THF (5 ml) was added 1.6 M *n*-BuLi in hexane (8.23 mL, 13.2 mmol) at −78˚C. The resulting mixture was stirred for 20 min at −78˚C, *N*-(diphenylmethylene)glycine methyl ester (**20**, 2.77 g, 10.9 mmol) was added and stirring was continued for 20 min. Next, HMPA (2.29 mL, 13.2 mmol) and TBDPS-propargyl bromide (**19**, 7.80 g, 21.9 mmol) were added and the reaction was stirred for 3 hours. It was poured into saturated NH_4Cl and the product was extracted with Et_2O (3 ×). The combined organic layers were washed with saturated aqueous NaCl, dried (Na_2SO_4) and evaporated *in vacuo*. The product was purified by flash column chromatography affording **23** (4.38 g, 8.28 mmol, 76%). R*f* = 0.6 (EtOAc/heptanes, 1/2). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.73−7.67 (m, 6H), 7.44−7.27 (m, 7H), 7.24−7.08 (m, 7H), 4.39 (dd, *J* = 8.8, 4.6 Hz, 1H), 3.72 (s, 3H), 3.14−3.01 (m, 2H), 1.02 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 139.5, 136.0, 135.7, 135.7, 133.6, 133.6, 130.6, 129.4, 129.4, 129.3, 128.7, 128.5, 128.3, 128.2, 127.7, 127.7, 107.7, 81.9, 64.2, 52.5, 27.2, 25.4, 18.6. HRMS (ESI) *m/z* calculated for $C_{35}H_{36}NO_2Si$ $(M+H)^+$: 530.2515, found: 530.2507.

N-(Diphenylmethylene)-(3-trimethylsilylpropargyl)glycine *tert*-butyl ester (24)



To a mixture of *N*-(diphenylmethylene)glycine *tert*-butyl ester (**21**, 297 mg, 1 mmol), tetrabutylammonium bromide (35 mg, 0.1 mmol) and TMS-propargyl bromide (**18**, 956 mg, 5 mmol) in toluene/THF (4 ml, 7:3) was added KOH (1.5 mL, 50% aqueous) at −40˚C. The resulting mixture was stirred for 8 hours at −40˚C, water was added and the product was extracted with Et_2O (2 ×). The combined organic layers were washed with saturated aqueous NaCl, dried (Na_2SO_4) and evaporated *in vacuo*. The product was purified by flash column chromatography affording **24** (405 mg, 1.00 mmol, >99%). R*f* = 0.72 (EtOAc/heptanes, 1/5). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.65−7.63 (m, 2H), 7.44−7.27 (m, 6H), 7.19−7.18 (m, 2H), 4.17 (dd, *J* = 8.2 Hz, 1H), 2.91−2.77 (m, 2H), 1.44 (s, 9H), 0.10 (s, 9H). Spectral data are in accordance with literature.[24]

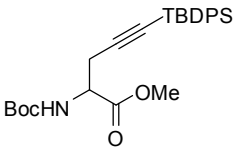
2-(*tert*-Butoxycarbonylamino)-5-(trimethylsilyl)pent-4-ynoic methyl ester (26)



To a solution of *N*-(diphenylmethylene)trimethylsilanepropargylglycine methyl ester (**22**, 2.02 g, 5.58 mmol) in Et_2O (30 mL) was added 1 M aqueous HCl (5 mL). Next, the reaction mixture was stirred for 72 h at room temperature, H_2O (10 mL) was added and the reaction mixture was washed with DCM (3 ×). Subsequently, saturated aqueous $NaHCO_3$ was added and the product was extracted with DCM (3 ×), dried (Na_2SO_4), filtrated and concentrated *in vacuo* to afford the HCl-salt. To a solution of the HCl salt (1.31 g, 5.57 mmol) in DCM (15 mL) was added Boc_2O (2.43 g, 11.14 mmol) and Et_3N (1.54 mL, 11.1 mmol). The reaction mixture was stirred overnight at room temperature and subsequently acidified to

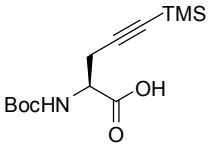
pH 4 (5% $KHSO_4$) and the product was extracted with DCM (3 ×). The combined organic layers were washed with saturated aqueous NaCl, dried (Na_2SO_4) and evaporated *in vacuo*. The product was purified by flash column chromatography affording **26** (1.41 g, 4.72 mmol, 85%). R*f* = 0.53 (EtOAc/heptanes, 1/2). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.48−4.40 (m, 1H), 3.76 (s, 3H), 2.78 (dd, *J* = 17.0, 4.8 Hz, 1H), 2.70 (dd, *J* = 17.0, 4.8 Hz, 1H), 1.46 (s, 9H), 0.14 (s, 9H). Spectral data are in accordance with literature.[25]

Methyl 2-(*tert*-butoxycarbonylamino)-5-(*tert*-butyldiphenylsilyl)pent-4-ynoate (27)



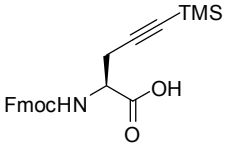
A solution of *N*-(diphenylmethylene)-(3-*tert*-butyldiphenylsilylpropargyl)glycine methyl ester **23** (2.00 g, 3.78 mmol) in Et_2O (20 mL) was treated with 1 M aqueous HCl (35 mL). Next, the reaction mixture was stirred for 72 h at room temperature, H_2O (10 mL) was added and the reaction mixture was washed with DCM (3 ×). Next, saturated $NaHCO_3$ was added and the product was extracted with DCM (3 ×), dried (Na_2SO_4), filtrated and concentrated *in vacuo* to afford the HCl-salt. To a solution of the HCl-salt (1.33 g, 3.64 mmol) in DCM (15 mL) was added Boc_2O (1.59 g, 7.28 mmol) and Et_3N (1.01 mL, 7.28 mmol). The reaction mixture was stirred overnight at room temperature and subsequently acidified to pH 4 (5% $KHSO_4$) and the product was extracted with DCM (3 ×). The combined organic layers were washed with saturated aqueous NaCl, dried (Na_2SO_4) and evaporated *in vacuo*. The product was purified by flash column chromatography affording **27** (1.28 g, 2.75 mmol, 73%). R*f* = 0.59 (EtOAc/heptanes, 1/2). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.79−7.73 (m, 4H), 7.42−7.32 (m, 6H), 5.41 (d, *J* = 7.3 Hz, 1H), 4.65−4.46 (m, 1H), 3.76 (s, 3H), 2.96 (d, *J* = 4.4 Hz, 2H), 1.45 (s, 9H), 1.07 (s, 9H). HRMS (ESI) *m/z* calculated for $C_{27}H_{35}NO_4Si$ $(M+H)^+$: 488.2233, found: 488.2211.

(*S*)-2-(*tert*-Butoxycarbonylamino)-5-(trimethylsilyl)pent-4-ynoic acid (28)



To a suspension of *N*-Boc protected amino acid methyl ester **26** (688 mg, 2.30 mmol) in *t*-amylalcohol (6.9 mL) was added 1 M $KHCO_3$ (6.9 mL) and aqueous *alcalase* solution (5.5 mL). The reaction mixture was stirred vigorously at 35 ˚C for 18 hours. Next, *alcalase* (1.4 mL) was added and the reaction mixture was stirred for another 6 hours, water was added and the mixture was washed with DCM (2 ×), subsequently acidified to pH 4 (5% $KHSO_4$) and the product was extracted with EtOAc (3 ×). The combined organic layers were dried (Na_2SO_4) and evaporated *in vacuo* affording **28** (0.31 g, 1.08 mmol, 47%). R*f* = 0.20 (1% acetic acid in EtOAc/heptanes, 1/2). FTIR (film): ν 3442, 2958, 2180, 1692, 1160 cm^{-1}. ^{1}H NMR (400 MHz, D_2O): δ = 4.48−4.45 (m, 1H), 2.85-2.73 (m, 2H), 1.47 (s, 9H), 0.15 (s, 9H). ^{13}C NMR (75 MHz, CD_3OD): δ = 174.3, 157.4, 103.2, 87.7, 80.6, 53.8, 28.7, 24.2, 0.3. HRMS (ESI) calculated for $C_{13}H_{23}NO_4Si$ $(M-H)^-$: 284.0968, found: 284.0946.

(*S*)-2-(((9*H*-Fluoren-9-yl)methoxy)carbonylamino)-5-(trimethylsilyl)pent-4-ynoic acid (32)

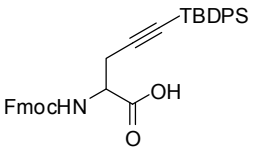


To a solution of **27** (240 mg, 0.84 mmol) in DCM (5 mL) was added TFA (5 mL) and the reaction mixture was stirred for 30 min at room temperature. Toluene was added and solvents were removed *in vacuo* affording the crude TFA salt.

Next, the (*S*)-2-amino-5-(trimethylsilyl)pent-4-ynoic acid TFA salt (226 mg, 0.80 mmol) was resuspended in H_2O/MeCN (20 mL, 1:1) and the pH was adjusted to 8.5

(Et_3N). Then, a solution of Fmoc-succinimide (257 mg, 0.762 mmol) in MeCN (20 mL) was added and the mixture was stirred for 4 h at pH 8.5. Subsequently, the mixture was acidified to pH 2.7 (1 M aqueous HCl), the organic layer was evaporated *in vacuo,* and the product was extracted with EtOAc (3 ×). The combined organic layers were washed with water (pH 4), dried (Na_2SO_4) and evaporated *in vacuo* affording **32** (325 mg, 0.80 mmol, 95 %). R*f* = 0.3 (EtOAc/heptanes, 1/2). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.79−7.76 (m, 2H), 7.64−7.59 (m, 2H), 7.43−7.39 (m, 2H), 7.35−7.30 (m, 2H), 4.58−4.54 (m, 1H), 4.42 (t, *J* = 6.6 Hz, 2H), 4.26 (t, *J* = 7.2 Hz, 1H), 2.86−2.83 (m, 2H), 0.17 (s, 9H).

2-(((9*H*-Fluoren-9-yl)methoxy)carbonylamino)-5-(*tert*-butyldiphenylsilyl)pent-4-ynoic acid (33)



To a solution of **28** in THF (5.7 mL) was added 0.15 M LiOH in H_2O (5.7 mL, 0.86 mmol) and the mixture was stirred for 4 h at room temperature. The organic solvent was removed *in vacuo*, 5% $KHSO_4$ was added and the free acid was extracted with EtOAc (3 ×). The combined organic layers were dried (Na_2SO_4), and concentrated *in vacuo*. Subsequently the free acid was redissolved in DCM (2 mL), TFA (2 mL) was added and the reaction mixture was stirred for 30 min at room temperature. Toluene was added and solvents were removed *in vacuo* affording the crude TFA salt (395 mg, 0.86 mmol, 100%). Next, part of the (*R/S*)-2-amino-5-(*tert*-butyldiphenylsilyl)pent-4-ynoic acid TFA salt (226 mg, 0.80 mmol) was resuspended in H_2O/MeCN (10 mL, 1/1) and the pH was adjusted to 8.5 (Et_3N). Then, a solution of Fmoc-succinimide (274 mg, 0.817 mmol) in MeCN (8.3 mL) was added and the mixture was stirred for 3 h at pH 8.5. Subsequently the mixture was acidified to pH 2.3 (1 M aqueous HCl), the organic layer was evaporated *in vacuo,* and the product was extracted with EtOAc (3 ×). The combined organic layers were washed with water (pH 4), dried over Na_2SO_4 and concentrated *in vacuo*. Purification *via* flash column chromatography afforded **33** (311 mg, 0.52 mmol, 63%). R*f* = 0.2 (1% acetic acid in EtOAc/heptane, 1/2). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.79−7.76 (m, 6H), 7.63−7.60 (m, 2H), 7.41−7.31 (m, 8H), 7.24−7.20 (m, 2H), 4.52−4.48 (m, 1H), 4.32−4.29 (m, 2H), 4.19−4.15 (m, 1H), 3.08−2.89 (m, 2H), 1.03 (s, 9H). ^{13}C NMR (75 MHz, CD_3OD): δ = 173.8, 158.3, 145.2, 142.5, 136.7, 134.6, 130.6, 128.8, 128.2, 126.3, 120.9, 107.8, 83.5, 68.3, 54.5, 27.6, 24.4, 19.3. HRMS (ESI) *m/z* calculated for $C_{36}H_{35}NO_4Si$ $(M+H)^+$: 596.2233, found 596.2253.

General procedure A for the synthesis of the peptides.
Loading of the trityl resin, synthesis of the pentapeptides, and capping, were carried out following standard Fmoc peptide chemistry protocols, involving HOBt/DIPCDI-mediated couplings and piperidine-induced Fmoc deprotections. Side-chain protection was *tert*-butyl (^{t}Bu) for serine.

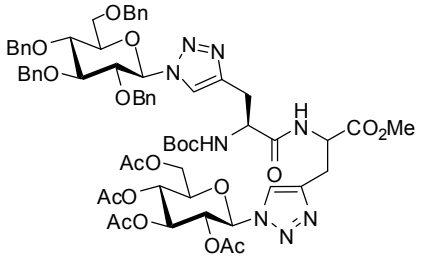
General procedure B for the cleavage from the resin.
The resin was treated with TFA:DCM (1:100) and shaken for 10 min. Subsequently, the resin was filtrated and the filtrate was neutralized with a 1:1 pyridine/methanol mixture. This procedure was repeated until TLC analysis indicated the absence of free amine in the filtrate. The combined organic layers were evaporated *in vacuo* affording the peptide.

General procedure C for the cycloaddition in solution.
To a solution of the peptide (1 equiv) and glycoside (1 equiv) in THF (0.25 M) was added CuI (1 equiv) and TBTA (1 equiv). Next, the mixture was stirred for 24 h at room temperature, $KHSO_4$ (5% aqueous) was added and the product was extracted with EtOAc (3 ×). The combined organic layers were washed with saturated aqueous $NaHCO_3$, brine, dried (Na_2SO_4) and evaporated *in vacuo*. The product was purified by flash column chromatography using EtOAc/heptane mixtures or by preparative TLC using butanol/H_2O/acetic acid (4/1/1).

General procedure D for the cycloaddition in solution:
To a solution of the peptide (1 equiv) and the glycoside (1 equiv) in *tert*–butanol (0.03 M) was added a mixture of $Cu(OAc)_2$ (20 mol%), sodium ascorbate (40 mol%) and TBTA (20 mol%) in H_2O (0.006 M and 0.013 M, respectively). The reaction was stirred for 24 h at room temperature, $KHSO_4$ (5% aqueous) was added and the product was extracted with EtOAc (3 ×). The combined organic layers were washed with saturated aqueous $NaHCO_3$, brine, dried (Na_2SO_4) and evaporated *in vacuo*. The product was purified by preparative TLC using butanol/H_2O/acetic acid (4/1/1).

General procedure E for the resin bound cycloaddition.
To the immobilized peptide (1 equiv) was added the glycoside (2 equiv), CuI (1 equiv), TBTA (1 equiv) followed by the addition of THF and DiPEA (1 equiv). Next, the mixture was shaken or rolled for 24 h at room temperature, subsequently the resin was washed with THF (3 ×), MeOH (1 ×) and dried.

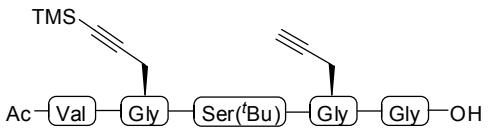
Boc-T4M(1-[β-D-Glc(Bn)$_4$]-T4M(1-[β-D-Glc(Ac)$_4$]-OMe (29)



To a solution of Boc-Pgl-Pgl(TMS)-OMe (**28**, 103 mg, 0.26 mmol) and tetra-*O*-benzyl azidoglucose (149 mg, 0.26 mmol) in *tert*–butanol (4 mL) was added a mixture of $Cu(OAc)_2$ (16 mg, 21 mol%), sodium ascorbate (21 mg, 41 mol%) in H_2O (4 mL). The reaction was stirred for 24 h at room temperature, $KHSO_4$ (5% aqueous) was added and the product was extracted with EtOAc (3 ×). The combined organic layers were washed with saturated aqueous $NaHCO_3$, brine, dried (Na_2SO_4) and evaporated *in vacuo*. Next, the monoglycosylated peptide was dissolved in MeOH (4 mL) and a catalytic amount of K_2CO_3 was added. The mixture was stirred for 4 h, amberlite IR 120 plus was added until the solution was neutral, and the ion exchange was filtered off. Evaporation of the solvent *in vacuo* afforded the deprotected peptide. A subsequent cycloaddition and workup using tetra-*O*-acetylazidoglucose, followed by purification *via* flash column chromatography afforded **29** (264 mg, 0.20 mmol, 77%). FTIR (ATR): ν = 3360, 1744, 1218 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.66 (s, 1H), 7.54 (s, 1H), 7.31−7.11 (m, 18H), 6.93−6.91 (m, 2H), 5.78−5.75 (m, 1H), 5.68 (d, *J* = 7.2 Hz, 1H), 5.58 (d, *J* = 9.0 Hz, 1H), 5.36 (dd, *J* = 6.6 Hz, 2H), 5.25−5.21 (m, 1H), 4.88−4.79 (m, 3H), 4.71 (d, *J* = 6.1 Hz, 1H), 4.57−4.39 (m, 6H), 4.27 (dd, *J* = 12.5 Hz, 1H), 4.09−4.06 (m, 1H), 4.02−3.92 (m, 2H), 3.89 (ddd, *J* = 9.9, 4.3, 1.5 Hz, 1H), 3.80−3.74 (m, 2H), 3.73−3.62 (m, 3H), 3.66 (s, 3H), 3.28−3.08 (m, 4H), 2.01 (s, 3H), 1.97 (s, 3H), 1.96 (s, 3H), 1.80 (s, 3H), 1.36 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.1, 171.1, 170.6, 170.0, 169.4, 169.3, 155.7, 143.6, 143.3, 138.3, 137.9, 137.9, 137.2,

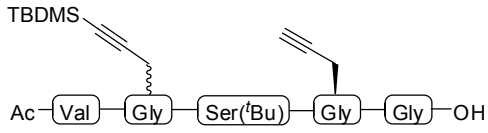
128.6, 128.5, 128.5, 128.3, 128.1, 128.0, 127.9, 127.9, 127.8, 122.0, 121.1, 87.6, 85.8, 85.6, 81.0, 80.3, 78.0, 77.4, 75.9, 75.3, 75.2, 74.9, 73.6, 72.7, 70.7, 68.6, 67.8, 61.6, 53.7, 52.7, 51.9, 28.4, 27.9, 27.8, 20.7, 20.7, 20.6, 20.2. HRMS (ESI) *m/z* calculated for $C_{64}H_{77}N_8O_{19}$ $(M+H)^+$: 1261.5305, found: 1261.5354.

Ac-Val-(*S*)-Pgl(TMS)-Ser(O^{t}Bu)-(*S*)-Pgl-Gly-OH (35a)



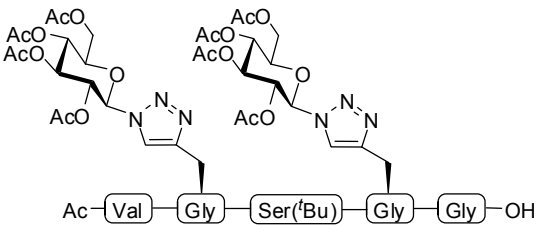
Preparation according to general procedures A and B for the synthesis of the peptides and cleavage from the resin afforded **35a** (19 mg, 0.03 mmol, 64%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.42 (d, *J* = 7.4, 1H), 4.61−4.56 (m, 1H), 4.53−4.50 (m, 1H), 4.43−4.38 (m, 1H), 4.24−4.18 (m, 1H), 4.00−3.88 (m, 2H), 3.72−3.58 (m, 2H), 2.80−2.77 (m, 1H), 2.75−2.73 (m, 1H), 2.70− 2.67 (m, 1H), 2.36−2.33 (m, 1H), 2.14−2.07 (m, 1H), 2.01 (s, 3H), 1.20 (s, 9H), 0.99 (dd, *J* = 6.7, 4.5 Hz, 6H), 0.11 (s, 9H). HRMS (ESI) *m/z* calculated for $C_{33}H_{47}N_5O_9Si$ $(M+H)^+$: 644.3092, found: 644.3046.

Ac-Val-Pgl(TBDPS)-Ser(O^{t}Bu)-(*S*)-Pgl-Gly-OH (35b)



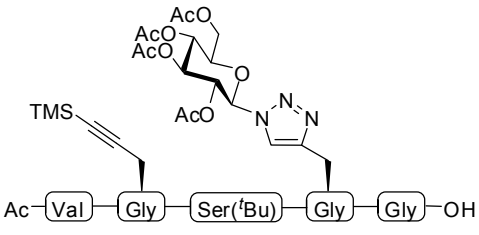
Preparation according to general procedures A and B for the synthesis of the peptides and cleavage from the resin afforded **35b** (25 mg, 0.03 mmol, 59%). ^{13}C NMR (75 MHz, CD_3OD): δ = 174.2, 174.0, 173.2, 172.3, 172.0, 171.9, 136.6, 136.6, 134.4, 134.4, 130.5, 130.5, 128.7, 128.7, 107.8, 107.5, 83.3, 83.2, 80.6, 80.3, 75.1, 74.9, 72.2, 72.0, 62.4, 62.2, 60.6, 60.2, 56.1, 55.6, 54.1, 54.0, 53.6, 53.4, 32.2, 31.6, 27.7, 27.7, 27.6, 22.6, 22.6, 22.5, 22.3, 19.9, 19.9, 18.8, 18.7. HRMS (ESI) *m/z* calculated for $C_{42}H_{57}N_5O_8Si$ $(M+H)^+$: 832.3693 found: 832.3686.

Ac-Val-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-Ser(^{t}Bu)- (*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-Gly-OH (39a)



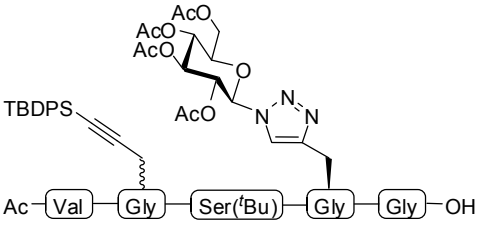
Preparation from resin bound PG-Val-Pgl(TMS)-Ser(O^{t}Bu)-Pgl-Gly-OH (**35a**) similar to the general procedure E for the resin bound cycloaddition using azido saccharide as glycoside, with the exception of using DMF instead of THF as solvent. Subsequent cleavage from the resin afforded **39a** (53 mg, 0.041 mmol, 40%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.29 (d, *J* = 7.8 Hz, 1H), 8.24 (d, *J* = 7.8 Hz, 1H), 8.18 (d, *J* = 28.0 Hz, 2H), 8.09 (d, *J* = 6.8 Hz, 1H), 7.96 (t, *J* = 5.9 Hz, 1H), 6.10 (dd, *J* = 9.1, 2.8 Hz, 2H), 5.67 (t, *J* = 9.4 Hz, 1 H), 5.61 (t, *J* = 9.3 Hz, 1H), 5.56−5.49 (m, 2H), 5.30 (dt, *J* = 9.5, 2.8 Hz, 2H), 4.41 (dd, *J* = 12.4, 5.7 Hz, 1H), 4.33−4.16 (m, 8H), 3.95 (d, *J* = 5.7 Hz, 2H), 3.66 (d, *J* = 0.4 Hz, 4H), 2.06 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.97 (s, 3H), 1.17 (s, 9H), 0.93 (dd, *J* = 6.7, 2.1 Hz, 6H). ^{13}C NMR (75 MHz, CD_3OD): δ = 171.9, 171,7, 171.0, 170.9, 170.4, 169.6, 169.5, 169.4, 168.7, 143.3, 143.2, 134.8, 134.7, 127.2, 126.9, 121.9, 121.7, 84.6, 73.9, 73.2, 72.3, 72.2, 70.2, 70.1, 67.4, 67.3, 66.3, 61.2, 60.6, 58.7, 57.4, 57.1, 54.1, 52.2, 51.9, 40.1, 39.5, 36.0, 29.8, 28.9, 28.8, 26.8, 26.6, 20.7, 18.8, 18.7, 18.4, 17.9, 17.4, 16.9, 16.5. HRMS (ESI) *m/z* calculated for $C_{54}H_{77}N_{11}O_{26}$ $(M+H)^+$: 1340.4758, found: 1340.4720.

Ac-Val-(*S*)-Pgl(TMS)-Ser(*t*Bu)- (*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-Gly-OH (38a)



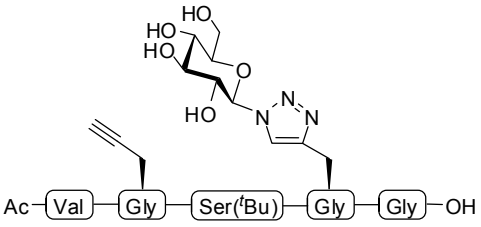
Preparation from PG-Val-Pgl(TMS)-Ser(O*t*Bu)-Pgl-Gly-OH (**35a**) according to general procedure C for the cycloaddition in solution afforded **38a** (19 mg, 0.019 mmol, 34%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.15 (s, 1H), 6.10 (d, *J* = 9.1 Hz, 1H), 5.60 (t, *J* = 9.3 Hz, 1H), 5.52 (t, *J* = 9.4 Hz, 1H), 5.28 (t, *J* = 9.6 Hz, 1H), 4.67 (dd, *J* = 8.8, 4.4 Hz, 1H), 4.51 (dd, *J* = 9.9, 4.4 Hz, 1H), 4.41 (t, *J* = 5.8 Hz, 1 H), 4.30 (dd, *J* = 12.0, 4.6 Hz, 1 H), 4.25−4.15 (m, 4H), 3.78−3.73 (m, 2H), 3.71−3.66 (m, 2H), 3.61−3.57 (m, 1H), 3.22−3.14 (m, 1H), 2.92−2.74 (m, 2H), 2.06 (s, 3H), 2.04 (s, 3H), 2.04 (s, 3H), 2.00 (s, 3H), 1.83 (s, 3H), 1.17 (s, 9H), 1.00 (dd, *J* = 6.8, 3.6 Hz, 6H), 0.11 (s, 9H). ^{13}C NMR (75 MHz, CD_3OD): δ = 174.3, 174.0, 172.5, 172.4, 172.3, 172.0, 171.5, 171.3, 170.5, 123.6, 104.1, 87.6, 86.6, 75.9, 75.3, 74.2, 72.1, 69.3, 63.1, 62.6, 61.1, 55.8, 54.6, 54.2, 31.8, 30.8, 28.5, 27.8, 22.9, 22.7, 20.7, 20.7, 20.6, 20.2, 20.0, 19.1, 0.2. HRMS (ESI) *m/z* calculated for $C_{43}H_{66}N_8O_{17}Si$ $(M+H)^+$: 995.4393, found: 995.4407.

Ac-Val-Pgl(TBDPS)-Ser(*t*Bu)- (*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-Gly-OH (45)



Preparation from PG-Val-Pgl(TBDPS)-Ser(O*t*Bu)-Pgl-Gly-OH (**35b**) according to general procedure C for the cycloaddition in solution afforded afforded **45** (12.9 mg, 0.01 mmol, 42%).
^{1}H-NMR (400 MHz, CD_3OD) δ = 7.79-7.70 (m, 5H), 7.38-7.30 (m, 6H), 4.66 & 4.63 (2 × dd, *J* = 7.9, 3.7 & 6.4, 3.7 Hz, 1H), 4.53 & 4.52 (2 × t, *J* = 7.9 & 8.1, 1H), 4.37 & 4.36 (2 × t, *J* = 4.1 & 4.5 Hz, 1H), 4.19 & 4.07 (2 × d, *J* = 6.8 & 7.1, 1H), 3.90-3.78 (m, 2H), 3.60 (ddd, *J* = 14.1, 9.3, 4.7 Hz, 1H), 3.48-3.38 (m, 1H), 3.06-2.98 (m, 1H), 2.90-2.76 (m, 1H), 2.73-2.55 (m, 2H), 2.24 & 2.23 (2 × t, *J* = 2.6 & 2.6 Hz, 1H), 2.04-1.94 (m, 1H), 1.93 (s, 2H), 1.86 (s, 2H),1.10 & 1.06 (2 × s, 9H), 0.99 (d, *J* = 5.6, 9H), 0.87 & 0.81 (2 × dd, *J* = 8.1, 7.0 & 8.7, 6.9 Hz, 6H. HRMS (ESI) *m/z* calculated for $C_{56}H_{77}N_8O_{17}Si$ $(M+H)^+$: 1161.5176, found: 1161.5217.

Ac-Val-(*S*)-Pgl-Ser(*t*Bu)- (*S*)-T4M(1-[β-D-Glc])-Gly-OH (40)

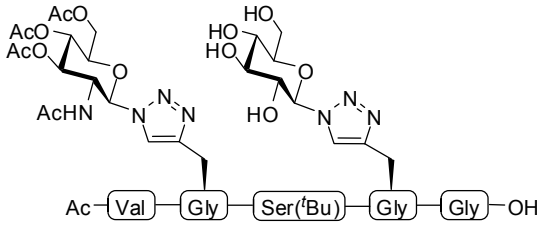


A) A solution of Ac-Val-Pgl(TBDPS)-Ser(*t*Bu)-4TA(1′-[β-D-Glc(Ac)$_4$])-Gly-OH (**45**, 12.9 mg, 0.01 mmol) in THF (2 mL) was treated with 1 M TBAF in THF (0.3 mL, 0.3 mmol) and stirred for 24 h at 40 °C. Completion of the reaction was evidenced by LRMS after 2 days.

B) To a solution of Ac-Val-Pgl(TMS)-Ser(*t*Bu)-4TA(1′-[β-D-Glc(Ac)$_4$])-Gly-OH (**38a**, 19 mg, 0.019 mmol) in MeOH (2mL) was added solid K_2CO_3 (excess) and the mixture was stirred overnight at room temperature. Amberlite IR 120 plus was added until the solution was neutral, and the ion exchange was filtered off. Evaporation of the solvent *in vacuo* afforded **40** (13.7 mg, 0.018 mmol, 96%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.31 (s, 1H), 5.90 (d, *J* = 9.2 Hz, 1H), 4.78 (t, *J* = 6.8 Hz, 1H), 4.65 (t, *J* = 5.6 Hz, 1H), 4.33 (d, *J* = 7.1 Hz, 1H), 4.17 (t, *J* = 9.1 Hz, 1H), 4.13−4.09 (m, 1H), 4.01−3.81 (m, 10H), 3.54 (dd, *J* = 15.3, 5.5 Hz, 1H), 3.48−3.41 (m, 1H), 2.92−2.90 (m, 2H), 2.63 (t, *J* = 2.5 Hz, 1H), 2.24 (s, 3H), 1.35 (s, 9H), 1.15 (d, *J* = 6.8 Hz, 6H). ^{13}C

NMR (75 MHz, CD_3OD): δ = 173.9, 173.3, 172.9, 171.2, 170.9, 170.7, 142.7, 122.9, 87.0, 78.9, 78.3, 75.4, 75.2, 74.7, 71.8, 68.4, 60.5, 59.9, 59.0, 53.7, 52.6, 51.6, 29.8, 26.7, 25.9, 21.2, 20.5, 17.9, 17.0. HRMS (ESI) *m/z* calculated for $C_{32}H_{50}N_8O_{13}$ $(M+H)^+$: 777.3395, found: 777.3396.

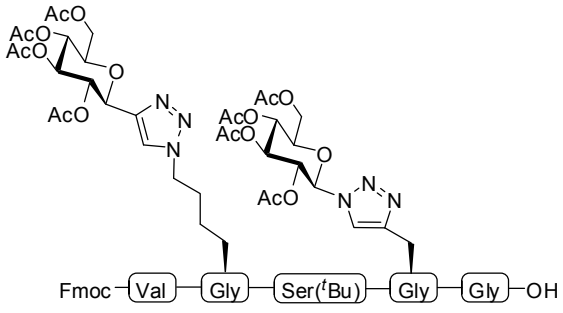
Ac-Val-(*S*)-T4M(1-[β-D-GlcNAc(Ac)$_3$])-Ser(^{t}Bu)- (*S*)-T4M(1-[β-D-Glc])-Gly-OH (42)



A) Preparation from Ac-Val-Pgl-Ser(^{t}Bu)-4TA(1'-[β-D-Glc])-Gly-OH (**40**) according to general procedure B for the cycloaddition in solution afforded **42** (18 mg, 0.016 mmol, 98%).

B) Preparation from resin bound PG-Val-Pgl(TMS)-Ser(O^{t}Bu)-Pgl-Gly-OH (**38a**) by first performing a cycloaddition on the resin (according to general procedure E) using azidoglucose as glycoside, followed by deprotection of the TMS-acetylene by treatment with 1 M TBAF in THF at room temperature for 7.5 h. Subsequently, a second cycloaddition applying azidoglucoseamine as glycoside (according to general procedure E) was performed and finally the peptide was cleaved from the resing according to general procedure B to afford **42** (13.8 mg, 0.011 mmol, 35%). HRMS (ESI) *m/z* calculated for $C_{46}H_{70}N_{12}O_{21}$ $(M+H)^+$: 1149.4676, found: 1149.4691.

Fmoc-Val-(*S*)-T1B(1-[β-D-Glc(Ac)$_4$])-Ser(^{t}Bu)- (*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-Gly-OH (44)



Preparation from resin bound **36** by applying the general procedure E for the resin bound cycloaddition twice (first using an azidosaccharide and subsequently an acetylenic saccharide as glycoside), followed by cleavage from the resing according to general procedure B afforded **44** (48 mg, 0.032 mmol, 32%). ^{1}H NMR (400 MHz, CD_3OD/DMSO-D6): δ = 7.90 (s, 1H), 7.88 (s, 1H), 7.78−7.72 (m, 2H), 7.51−7.35 (m, 6H), 6.22 (d, *J* = 8.7 Hz, 1H), 5.67−5.56 (m, 2H), 5.44 (t, *J* = 9.4 Hz, 1H), 5.35−5.28 (m, 2H), 5.16 (t, *J* = 9.7 Hz, 1H), 4.88 (d, *J* = 9.9 Hz, 1H), 4.83−4.71 (m, 3H), 4.46−3.99 (m, 11H), 3.95−3.90 (m, 2H), 3.66−3.51 (m, 2H), 3.28−3.09 (m, 2H), 2.64 (dq, *J* = 1.9, 1.6 Hz, 1H), 2.10 (s, 3H), 2.08 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.86 (s, 3H), 1.86 (s, 3H), 1.42−1.30 (m, 4H), 1.17 (s, 9H), 0.96 (bs, 6H). HRMS (ESI) *m/z* calculated for $C_{70}H_{91}N_{11}O_{27}Na$ $(M+Na)^+$: 1540.5984, found: 1540.6019.

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Anyone who has never made a mistake,
has never tried anything new.

-Albert Einstein

I love argument, I love debate. I don't expect anyone
just to sit there and agree with me, that's not their job.

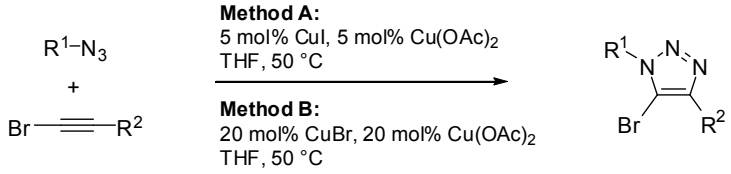
-Margaret Thatcher

Copper(I)-Mediated Synthesis of Trisubstituted [1,2,3]-Triazoles

CHAPTER 5

Abstract*:

A novel copper-catalyzed cycloaddition of bromoalkynes and organic azides is described. Copper-mediated coupling resulted in the formation of bromo-containing trisubstituted [1,2,3]-triazole derivatives in high yield and a regioselective manner, and was applied in the cycloaddition of a variety of bromoacetylenes and azides. The bromide substituent in the resulting triazoles provided a versatile synthetic handle for further functionalization to trisubstituted [1,2,3]-triazoles.



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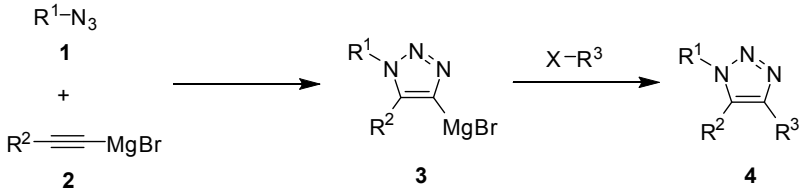
5.1 INTRODUCTION

For a long time, the [1,2,3]-triazole compound class received rather little appreciation among the nitrogen-containing aromatic heterocycles. Nevertheless, [1,2,3]-triazoles are versatile compounds, as reflected in the broad variety of applications including anticorrosive agents, dyes, agrochemicals and photographic materials.[1] Although no occurrences in Nature have been reported, [1,2,3]-triazoles feature in diverse pharmaceutical substances including anti-HIV[2] and anti-microbial[3] drugs as well as selective α_3-adrenergic receptor agonists.[4] Although the synthesis of [1,2,3]-triazoles had been studied for many years,[5] the recent discovery by the groups of Meldal and Sharpless that Cu(I)-salts catalyze the [3+2] cycloaddition between organic azides and acetylenes contributed significantly to the efficient preparation of 1,4-disubstituted-[1,2,3]-triazoles with exclusive regioselectively and in generally high yields.[6] As with most of the earlier synthetic procedures, however, the Meldal and Sharpless procedures are only appropriate for the preparation of disubstituted triazoles. Methods for the synthesis of trisubstituted triazoles are scarce, and are frequently hampered by a lack of regioselectivity or by a restriction in choice of functional groups.[7,8]

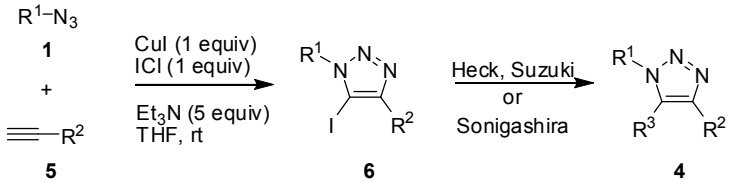
Two synthetic procedures for the regioselective synthesis of disubstituted triazoles deserve mentioning. Firstly, a strategy developed recently by Sharpless *et al.*[8] involves the synthesis of 1,5-disubstituted [1,2,3]-triazoles *via* the addition of bromomagnesium acetylides **2** to azides **1** (Scheme 1), and is applicable to a wide array of azides and alkynes. A useful feature of the strategy is the option to trap the magnesium intermediate **3** with a suitable electrophile, affording regioselectively the 1,4,5-trisubstituted [1,2,3]-triazoles **4**.



Scheme 1. *Trisubstituted [1,2,3]-triazoles via reaction of magnesium acetylides with azides.*

While in the above procedure the organic moieties of the acetylene and the azide end up in a 1,5-disposition in the resulting triazole, another strategy recently reported by Chen *et al.*[9] describes the preparation of 5-iodo-1,4-disubstituted [1,2,3]-triazoles

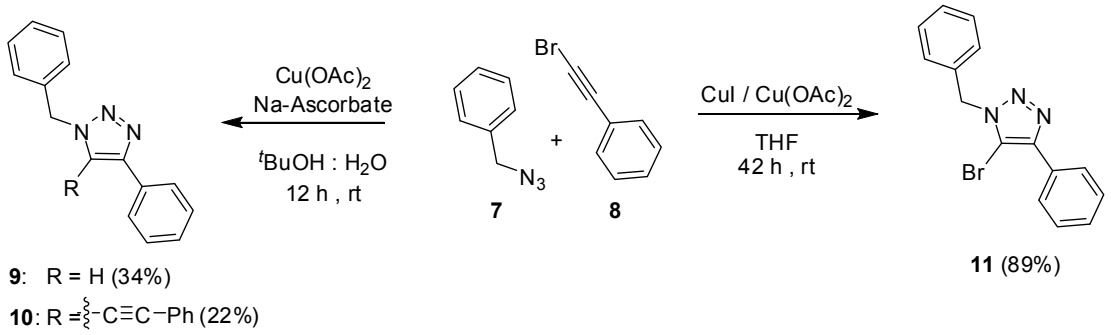
(Scheme 2). It was shown that subjecting an acetylene (**5**) and an azide (**1**) to a combination of stoichiometric amounts of CuI and ICl led to the corresponding desired triazoles in a one-pot reaction with yields averaging around 70% for a variety of substrates. The resulting 5-iodo-1,4-disubstituted triazoles could be further functionalized by several palladium-catalyzed cross-coupling reactions (*e.g.* Suzuki, Heck and Sonogashira reactions).



Scheme 2. *Trisubstituted [1,2,3]-triazoles via Cu(I)-mediated reaction of acetylenes and azides.*

Considering the fact that equimolar quantities of both copper-salts and iodine monochloride are required, we were prompted to further explore our finding that an organic azide and a bromoacetylene upon treatment with 20 mol% of $Cu(OAc)_2$ and sodium ascorbate in a mixture of *tert*-butyl alcohol and water, not only yielded a 1,4-disubstituted triazole lacking the bromide (*i.e.* the product normally formed from a terminal acetylene), but also a 1,4,5-trisubstituted triazole, as reported in Section 4.2.2.

To investigate the generality of such trisubstituted triazole formation from an azide and a (substituted) bromoacetylene, a model system consisting of benzyl azide **7** and phenylbromoacetylene **8** was subjected to "optimized" [3+2]-CuAAC conditions (Scheme 3) as described earlier (Chapter 3), leading to a mixture of the standard cycloadduct **9**, and a trisubstituted triazole product **10**. The formation of such a trisubstituted compound is attractive, thus attempts to improve the yield were examined. We envisioned that performing the reaction in an aprotic medium (*e.g.* THF), would disfavour the formation of **9** and could consequently lead to an improved yield of the trisubstituted triazole product **10**. As a first study, a 1:1 mixture of **7** and **8** in THF was subjected to 10 mol% of CuI, however, it was disappointing to find that nearly no reaction took place. Likewise, treatment of the substrates with 10 mol% of $Cu(OAc)_2$ failed to yield any trisubstituted product.



Scheme 3. *Initial experiments with a model system.*

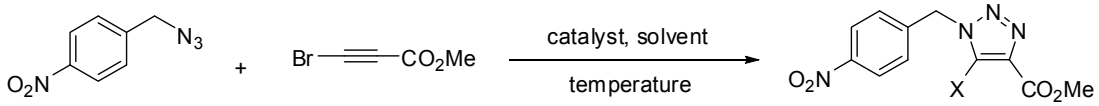
To our surprise, treatment of phenylbromoacetylene (**8**) and benzyl azide (**7**) with a mixture of CuI and $Cu(OAc)_2$ (5 mol% each, THF), resulted in the new 5-bromo-1,4–disubstituted triazole compound **11**. The specific regiochemistry of the new triazole was determined *via* reduction to the 5-hydrogen-[1,2,3]-triazole by subjection to ^{i}PrMgCl, followed by quenching with methanol, and spectral comparison of the formed product to the known 1,4-disubstituted triazole. Further study proved that besides the 5-bromotriazole, a small amount of 5-iodotriazole was formed as well.

In our opinion, the 5-bromosubstituted triazole **11** was even more interesting than the trisubstituted triazole **10**, taking into account that a bromide can act as a versatile handle for further functionalization reactions, such as Pd-catalyzed cross-coupling reactions, as recently described by Chen[10] for the iodotriazoles.

5.2 OPTIMIZATION OF REACTION CONDITIONS

Encouraged by the results achieved with phenylbromoacetylene and benzyl azide (**7** and **8**) we selected the less volatile *p*-nitrobenzyl azide (**12**) and methyl bromopropiolate (**13**) as model substrates for optimization studies, as summarized in Table 1. Entries 1 and 2 show that Cu-catalysis plays an essential role in the mechanism, while subjecting **12** and **13** to 10 mol% of CuI (entry 3) led to a 4:1 mixture of the bromo- and iodosubstituted 1,4-disubstituted triazoles **14** and **15**. The low yield (32%) of the reaction was effectively raised to 78% upon heating to 55 °C (entry 4). Heating the reaction mixture to temperatures higher than 50 °C, however, led to the formation of (small) amounts of the undesired 1,5-regioisomer, probably formed *via* thermal [3+2] cycloaddition.

Table 1. *Optimization of the [3+2] cycloaddition conditions.*



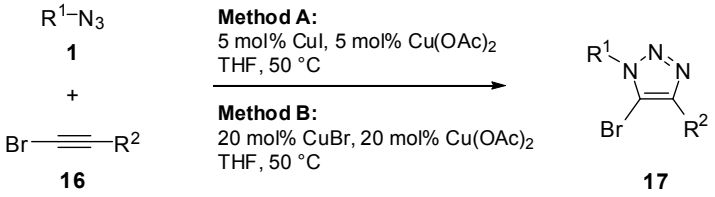
12 **13** **14**: X = Br **15**: X = I

entry	catalyst	loading (mol%)	solvent	T	t (h)	conv[a] (yield)[c]	**14:15**
1	-	-	THF	rt	22	trace	100:0
2	-	-	THF	55 °C	22	12%	100[b]:0
3	CuI	10	THF	rt	22	32%	80:20
4	CuI	10	THF	55 °C	22	78%	84[b]:16
5	$Cu(OAc)_2$	10	THF	rt	40	trace	n.d.
6	CuI/$Cu(OAc)_2$	5/5	THF	rt	16	66%	95:5
7	CuI/$Cu(OAc)_2$	5/5	MeCN	rt	16	<7%	n.d.
8	CuI/$Cu(OAc)_2$	5/5	Et_2O	rt	16	3%	n.d.
9	CuI/$Cu(OAc)_2$	5/5	DMF	rt	16	10%	n.d.
10	CuI/$Cu(OAc)_2$	5/5	toluene	rt	16	2%	n.d.
11	CuI/$Cu(OAc)_2$	5/5	DCM	rt	16	3%	n.d.
12	CuI/$Cu(OAc)_2$	5/5	THF	50 °C	16	>99% (99%)	94:6
13	CuI/$Cu(OAc)_2$	5/5	THF	reflux	16	>99% (95%)	93[b]:7
14	CuI/$Cu(OAc)_2$	5/5	THF	rt	40	97% (93%)	95:5
15	CuBr/$Cu(OAc)_2$	5/5	THF	rt	40	29%	100:0
16	CuBr/$Cu(OAc)_2$	20/20	THF	rt	40	56%[c]	100:0
17	CuBr/$Cu(OAc)_2$	20/20	THF	50 °C	16	>99% (97%)	100:0
18	CuI/$Cu(OAc)_2$	9/1	THF	rt	48	36%	n.d.
19	CuI/$Cu(OAc)_2$	1/9	THF	rt	48	<5%	n.d.
20	CuI + DIPEA	10	THF	rt	16	trace	n.d.
21	CuI/$PdCl_2$	5/5	THF	rt	48	trace	n.d.
22	CuI/$CuSO_4$	5/5	THF	rt	48	<5%	n.d.
23	CuBr/$Cu(OAc)_2$	5/5	THF	rt	48	29%	100:0
24	CuI/$CuCl_2$	5/5	THF	rt	48	12%	n.d.
25	CuCN/$Cu(OAc)_2$	5/5	THF	rt	48	<5%	n.d.
26	CuBr	10	THF	rt	48	<5%	100:0

[a]Conversion determined by ^{1}H-NMR. [b](Small) amounts of the regioisomer were also detected. [c]Isolated yields.

Much to our satisfaction, upon the combined action of CuI and $Cu(OAc)_2$, the conversion of substrates **12** and **13** greatly improved to 66% at room temperature (entry 6) and was driven to completion after prolonged reaction time or upon heating to 50 °C (entries 14 and 12). Next, we investigated the influence of other Cu-salts (*e.g.* CuCN, $Cu(OAc)_2$, $CuCl_2$, and CuBr), a mixture of CuI/$PdCl_2$ (entry 21), different relative catalyst loadings (entries 19 and 20) and solvents (*e.g.* DCM, DMF, MeCN, Et_2O and toluene, entries 7-11). In most cases, however, only small amounts of the desired product **14** could be isolated.

In cases that CuI was used, a small amount of the corresponding 5-iodotriazole **15** was also formed. To circumvent the formation of iodide **15**, a CuBr/$Cu(OAc)_2$ couple was used. As projected the 5-bromo-1,4-disubstituted triazole **14** was solely formed, albeit the reaction proceeded significantly slower (entry 15). In general, elevating the temperature (entries 6 *vs* 12 and 16 *vs* 17) and increasing the amount of catalyst (entry 15 *vs* 16) independently accelerated the reaction rate.

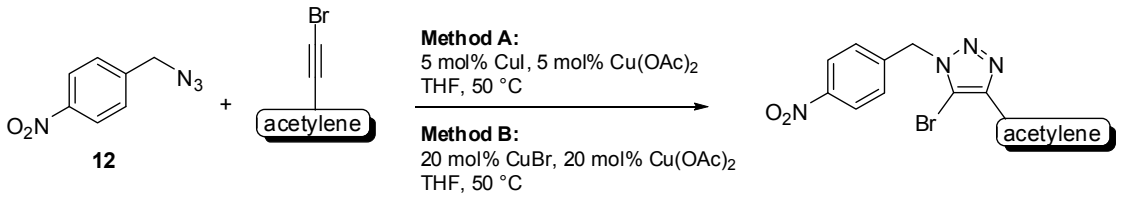


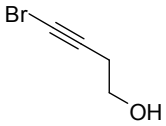
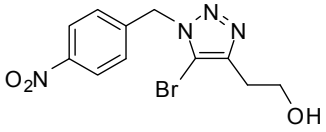
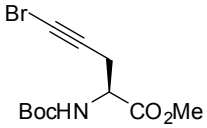
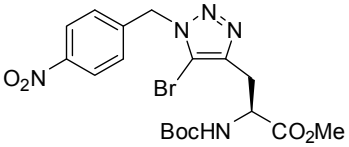
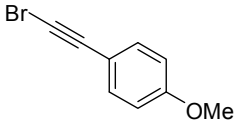
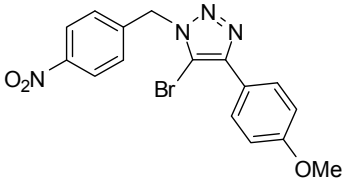
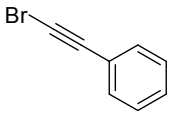
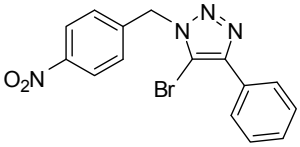
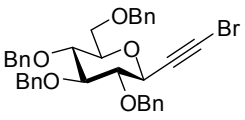
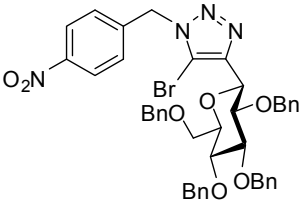
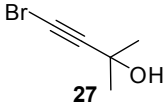
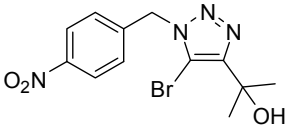
Scheme 4. *Optimized cycloaddition conditions.*

The optimal conditions that we eventually selected are shown in Scheme 4. With method A, reactions generally proceed faster, but the bromotriazole product is inevitably accompanied by small amounts of the 5-iodotriazole. Obviously, the formation of such by-products is hardly disadvantageous in case the halide substituent is further reacted to introduce other substituents such as in palladium-catalyzed cross-coupling reactions as reported by Chen *et al*.[10] Alternatively, in cases where a mixture of halide substituents is not desired, method B can be applied leading to the bromosubstituted triazole only.

5.3 SYNTHESIS OF 5-BROMO-[1,2,3]-TRIAZOLES

Table 2. *Variation of the bromoacetylene.*



entry	acetylene	product	yield
1	 **18**		**19a**: 85%[b] (16 h) **19b**: 92% (75 h)
2	 **20**		**21a**: 94%[a,b] (18 h) **21b**: 85% (76 h)
3	 **22**		**23a**: 85%[c,d] (50 h) **23b**: 84% (124 h)
4	 **8**		**24a**: 93%[c,d] (19 h) **24b**: 39% (19 h)
5	 **25**		**26a**: 63% (16 h)[b] **26b**: 98% (69 h)
6	 **27**		**28b**: 48% (50 h)

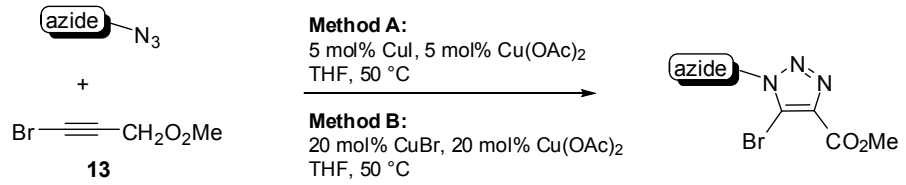
[a]Reactions performed at rt. [b]Isolated as a 95:5 Br:I mixture. [c]Isolated as a 84:16 Br:I mixture. [d]Reactions were carried out with 20 mol% of each catalyst.

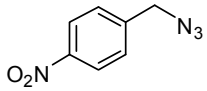
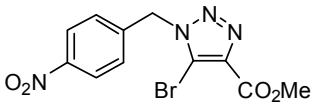
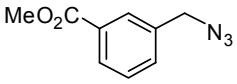
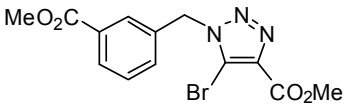
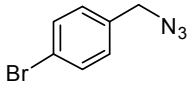
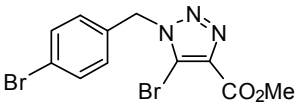
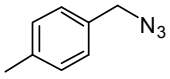
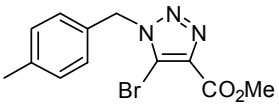
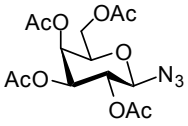
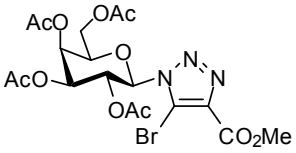
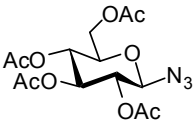
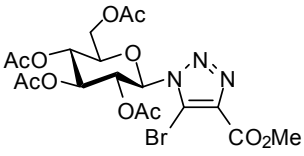
Having found two novel procedures for the [3+2] cycloaddition of bromoacetylenes, an array of triazoles was prepared by reaction of *p*-nitrobenzyl azide (**12**) with different bromoacetylenes, as summarized in Table 2. Bromoacetylenes are stable compounds, readily prepared in quantitative yields from acetylenes upon the action of NBS and catalytic $AgNO_3$. From Table 2, it becomes clear that the cycloaddition proceeds equally well for a range of different acetylenes including electron-donating or electron-withdrawing substituents. Yields are generally high, although a clear difference in reaction rate can be distinguished between the two methods. To our satisfaction, both methods were also applicable to sterically demanding acetylenic saccharides and amino acids as in the case of products **21a/b** and **26a/b** (entries 2 and 5).

Whereas earlier the azide starting material was kept constant, the coupling of methyl bromopropiolate (**13**) with a range of organic azides was also investigated, as displayed in Table 3. Clearly, a variety of different azides is well-tolerated, including sterically demanding azides (entries 5 and 6), resulting in the monosaccharide-substituted triazoles **36a/b** and **38a/b**. Furthermore, several substituted benzylic azides were also successfully applied, leading to triazoles **14, 30**, **32** and **34**. Again, it is clear that method B proceeds considerably slower than method A. However, by extension of reaction times, the CuBr-mediated reactions can generally be driven to completion as well.

At present, the mechanism of the [3+2] cycloaddition of bromoacetylenes and azides is unclear to us. One possibility is that only Cu(I)-species are involved, suggesting that the mechanism would proceed *via* Cu/Br-exchange, followed by cycloaddition and subsequent reaction of the Cu(I)-triazole complex with 'Br^+'. An alternative possibility involves an oxidative addition/reductive elimination cycle, in which initial oxidative addition of the bromoalkyne to Cu(I) leads to a Cu(III)-species,[11] followed by cycloaddition to a Cu(III)-triazole complex, and subsequent reductive elimination to the corresponding halotriazole.

Table 3. *Variation of the azide.*



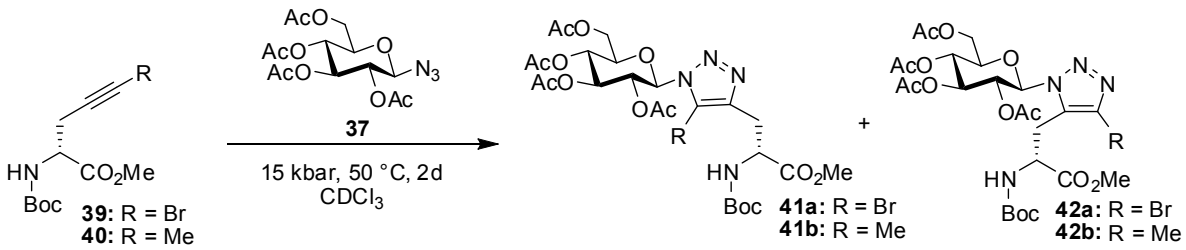
entry	azide	product	yield
1	 **12**		**14a**: 99%[a] (16 h) **14b**: 97% (16 h)
2	 **29**		**30a**: 81%[a,b] (16 h) **30b**: 99% (164 h)
3	 **31**		**32a**: 80%[a,b] (40 h)
4	 **33**		**34a**: 81%[a,b] (40 h)
5	 **35**		**36a**: 83%[a] (16 h) **36b**: 99% (140 h)
6	 **37**		**38a**: 67%[a] (16 h) **38b**: 91% (147 h)

[a]Isolated as a 95:5 Br:I mixture. [b]Reactions were performed at rt.

5.4 TRISUBSTITUTED [1,2,3]-TRIAZOLYL GLYCOPEPTIDES

Prior to our discovery that bromoacetylenes and organic azides can be ligated *via* a [3+2] cycloaddition, the synthesis of 5-bromotrisubstituted triazoles was already explored in the development of trisubstituted triazole-linked glycopeptides. In an early stage we envisioned that applying high pressure to substituted propargylglycines and azidosaccharides would lead to a mixture of 1,4- and 1,5-regioisomers.

Table 4. *High pressure-mediated cycloadditions.*



entry	R	yield of mixture	product	ratio (**41:42**)	product
1	Br	80%	**41a**	2:1	**42a**
2	Me	72%	**41b**	2:1	**42b**

In order to validate such an assumption, bromopropargylglycine **39** and methylpropargylglycine **40** were separately mixed with azidoglycoside **37** in deuterated chloroform and placed under 15 kbar at 50 °C for several days (Table 4). As anticipated, for both substrates a cycloaddition reaction occurred although very sluggishly, the reaction rate profile for formation of bromo-substituted triazole derivative (**41a/42a**) is depicted in Figure 1. It becomes clear that in the first 50 hours a relative fast reaction to 60% conversion took place, however, after that time the reaction rate dropped leading to 80% conversion after 175 hours. In addition, the methylpropargylglycine derivative **40** led to 72% conversion in a similar timeframe.

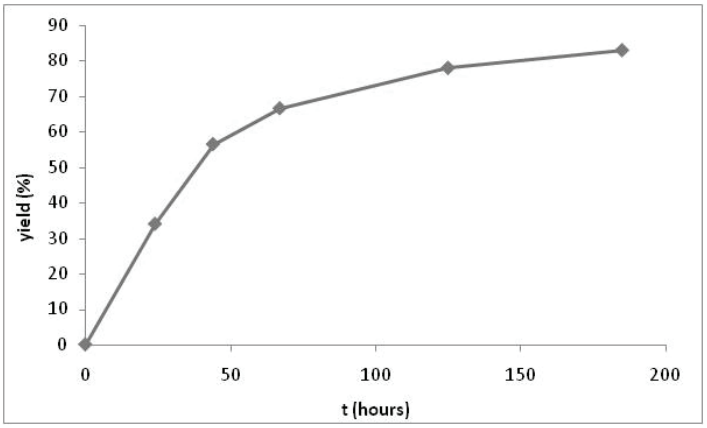
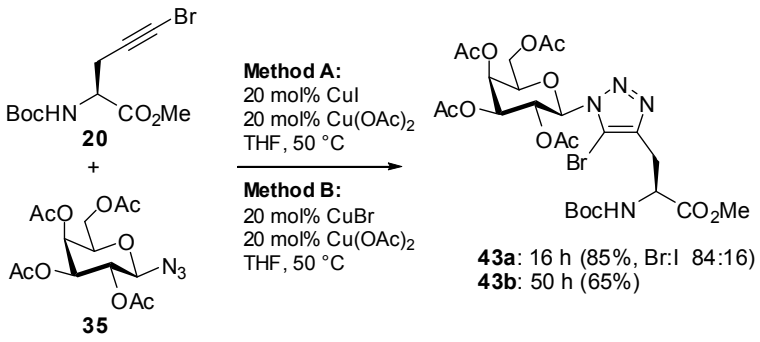


Figure 1. *Conversion over time for **41a/42a**.*

As expected, both 1,4- (**41**) and 1,5-regioisomers (**42**) were formed, in a 2:1 ratio respectively, with preference for the less sterically hindered 1,4-regioisomer. Unfortunately, separation of the regioisomers was futile. Therefore, also taking into consideration the very low reaction rate, the high pressure strategy for the formation of trisubstituted triazoles was abandoned.

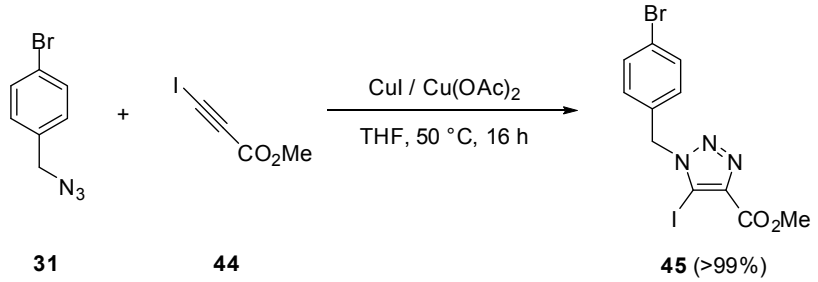
A more positive argument to explore other routes to trisubstituted triazoles came from our observation that halide-containing derivatives of triazoles can be synthesized in a stereoselective manner resulting in only the 1,4 regioisomer under copper(I)-catalysis. Thus, by application of the aforementioned methods A or B, bromo-substituted propargylglycine derivative **20** was coupled to azidogalactoside **35** (Scheme 5), resulting in acceptable yields of the glycosidic amino acid derivative **43**.



Scheme 5. *Synthesis of a 5-bromotriazole-linked glycoamino acid.*

5.5 CYCLOADDITION OF IODOACETYLENES

Since application of method A to bromoacetylenes inevitably led to the concomitant formation of small amounts of iodo-substituted triazoles, the possibility of preparing pure 5-iodotriazoles from the corresponding iodoacetylenes was also investigated. To this end, *p*-bromobenzyl azide (**31**) and methyl iodopropiolate (**44**) were subjected to a mixture containing 5 mol% CuI and $Cu(OAc)_2$ in THF at 50 °C. After 16 hours, full conversion of starting materials was observed, yielding the desired 5-iodo-1,4-disubstituted triazole **45** in excellent yield (Scheme 6).



31 **44** **45** (>99%)

Scheme 6. *Preparation of a 5-iodo-triazole.*

Unfortunately, the iodoacetylenes **46** and **47** (Figure 2) appeared too unstable to survive the reaction conditions, and condensing them with several azides failed to produce the desired iodotriazoles.

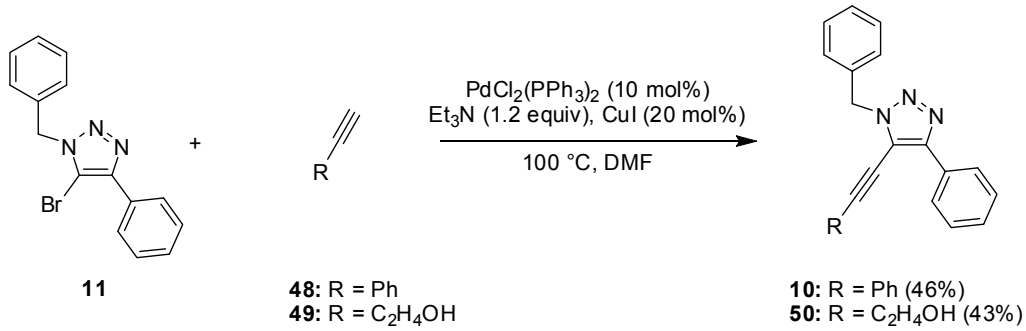
46 **47**

Figure 2. *Iodoacetylenes unable to undergo the cycloaddition.*

5.6 COUPLING REACTIONS

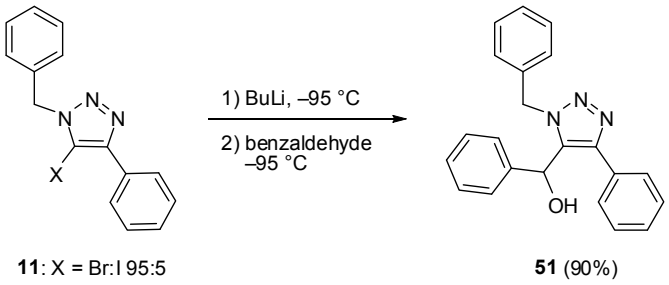
Various coupling reactions such as Suzuki couplings, Heck, and Sonogashira reactions were described by Chen *et al*.[10] for 5-iodotriazole-containing substrates. Applying several of these exact conditions to 5-bromotriazoles, however, was only partially successful. For example, Sonogashira reactions of 5-bromo-substituted

triazole **11** with acetylene **48** or **49** resulted in the formation of the desired products **10** and **50** in moderate yields of 46% and 43%, respectively (Scheme 7).



Scheme 7. *Sonogashira reaction with bromotriazole **11**.*

Even less rewarding were several attempts to functionalize the 5-bromo substrates by Heck reaction (e.g. $Pd(PPh_3)_2Cl_2$, K_2CO_3, THF, 80 °C) since the desired products were not observed at all. More successful was the, not by Chen *et al*. described, metal-halogen exchange of either halides (Br or I) under the action of BuLi at low temperature, followed by quenching with an appropriate electrophile such as benzaldehyde. Starting from **11**, the corresponding alcohol **51** was formed in high yield (Scheme 8). Nevertheless, given the fact that Chen *et al.* already investigated the further transformation of halotriazoles under a range of conditions, this line of research was not further explored.



Scheme 8. *Example of halogen-metal exchange.*

5.7 CONCLUSIONS

In conclusion, we have shown for the first time that the Cu-catalyzed cycloaddition between bromoalkynes and organic azides proceeds readily to form the corresponding 5-bromo-[1,2,3]-triazole derivatives in high yield and in a

regioselective manner. The procedure offers access to a variety of halide-containing trisubstituted triazoles, in particular with bromide, since various iodoacetylenes proved to be incompatible with the required reaction conditions. In cases where a mixture of halide substituents is undesirable, method B ($CuBr/Cu(OAc)_2$) can be applied leading solely to the bromo-substituted triazoles. The copper-catalyzed cycloaddition of bromoacetylenes and azides can be smoothly applied in the preparation of sterically demanding, trisubstituted [1,2,3]-triazolyl glycoamino acids. Finally, the halogen substituent of the resulting trisubstituted substrates offers a range of synthetic applications *via* metal-mediated derivatization.

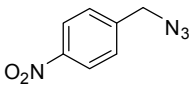
5.8 ACKNOWLEDGMENT

G. C. T. Dijkmans is acknowledged for his contribution to this chapter.

5.9 EXPERIMENTAL

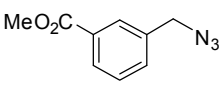
General procedure A for the azide formation.
To the bromine containing substrate (1 equiv) in MeCN (0.5 M) was added NaN_3 (1.7 equiv) and the mixture was stirred overnight at room temperature. Next, the mixture was concentrated *in vacuo* and subsequently taken up in DCM. The organic layer was washed with water, saturated aqueous $NaHCO_3$, brine, dried ($MgSO_4$) and evaporated *in vacuo*. Purification of the products was achieved *via* flash column chromatography.

p-Nitrobenzyl azide (12)



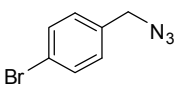
Preparation according to the general procedure A for azide formation afforded **12** (1.06 g, 5.91 mmol, 99%) as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.25 (d, *J* = 8.7 Hz, 2H), 7.52−7.49 (m, 2H), 4.51 (s, 2H). Spectral data are in accordance with literature.[12]

3-Azidomethyl benzoic acid methyl ester (29)



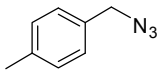
Preparation according to the general procedure A for azide formation afforded **29** (1.27 g, 6.64 mmol, >99%) as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.03−7.99 (m, 2H), 7.54−7.45 (m, 2H), 4.41 (s, 2H), 3.93 (s, 3H). Spectral data are in accordance with literature.[13]

***p*-Bromobenzyl azide (31)**



Preparation according to the general procedure A for azide formation afforded **34** (1.27 g, 5.89 mmol, 96%) as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.52 (d, *J* = 8.4 Hz, 2H), 7.20 (d, *J* = 8.6 Hz, 2H), 4.31 (s, 2H). Spectral data are in accordance with literature.[14]

_p_-Methylbenzyl azide (33)

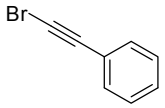


Preparation according to the general procedure A for azide formation afforded **36** (1.01 g, 5.70 mmol, 94%) as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.20 (ap d, *J* = 1.3 Hz, 2H), 4.29 (s, 2H), 2.36 (s, 3H). Spectral data are in accordance with literature.[15]

General procedure B for the preparation of the acetylenic bromides.

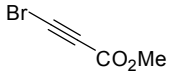
To a solution of the acetylene (1 equiv) in acetone (0.5 M) were added $AgNO_3$ (5 mol%) and *N*-bromosuccinimide (1.2 equiv) and the mixture was stirred for 4 h and diluted with water. Next the product was extracted with DCM (2 ×), and the combined organic layers were washed with water, saturated aqueous $NaHCO_3$, brine, dried ($MgSO_4$) and evaporated *in vacuo* to afford the desired product.

Bromophenylacetylene (8)



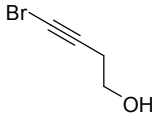
Preparation according to the general procedure B for the preparation of acetylenic bromides afforded **8** (177 mg, 0.87 mmol, 87%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.46−7.43 (m, 2H), 7.36−7.27 (m, 3H). Spectral data are in accordance with literature.[16]

Methyl 3-bromopropiolate (13)



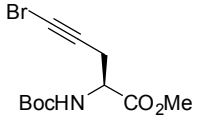
Preparation according to the general procedure B for the preparation of acetylenic bromides afforded **13** (873 mg, 4.66 mmol, 78%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.79 (s, 3H). Spectral data are in accordance with literature.[17]

4-Bromo-3-butynol (18)



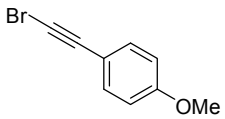
Preparation according to the general procedure B for the preparation of acetylenic bromides afforded **28** (788 mg, 3.26 mmol, 54%) as a liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.73 (t, *J* = 6.3 Hz, 2H), 2.49 (t, *J* = 6.3 Hz, 2H), 1.97 (br s, 1H). Spectral data are in accordance with literature.[18]

(*S*)-*N*-Boc-Bromopropargylglycine methyl ester (20)



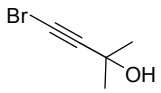
Preparation according to the general procedure B for the preparation of acetylenic bromides afforded **20** (155 mg, 0.51 mmol, 77%) as a yellowish solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.30 (br d, *J* = 6.8 Hz, 1H), 4.49−4.44 (m, 1H), 3.79 (s, 3H), 2.84−2.69 (m, 2H), 1.46 (s, 9H). Spectral data are in accordance with literature.[19]

4-Methoxyphenylbromoacetylene (22)



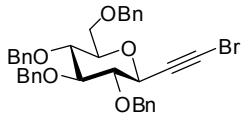
Preparation according to the general procedure B for the preparation of acetylenic bromides afforded **22** (212 mg, 0.97 mmol, 94%) as a yellowish solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.38 (d, *J* = 9.0 Hz, 2H), 6.83 (d, *J* = 9.0 Hz, 2H), 3.81 (s, 3H). Spectral data are in accordance with literature.[20]

3-Bromo-1,1-dimethyl-2-propynol (27)



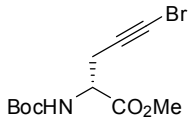
Preparation according to the general procedure B for the preparation of acetylenic bromides afforded **28** (148 mg, 0.76 mmol, 76%) as a liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.95 (br s, 1H), 1.52 (s, 6H). Spectral data are in accordance with literature.[21]

Tetra-*O*-benzyl-1-bromoacetylenic glucose (35)



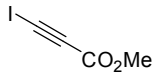
Preparation according to the general procedure B for the preparation of acetylenic bromides afforded **35** (115 mg, 0.17 mmol, 95%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.36−7.24 (m, 18H), 7.13−7.10 (m, 2H), 4.92 (dd, *J* = 10.4, 3.2 Hz, 2H), 4.82 (dd, *J* = 11.0, 7.4 Hz, 2H), 4.79 (d, *J* = 10.6, 1H), 4.57 (dd, *J* = 33.0, 12.2 Hz, 2H), 4.51 (d, *J* = 10.8), 4.06−4.04 (m, 1H), 3.74−3.65 (m, 2H), 3.62−3.58 (m, 3H), 3.43-3.39 (m, 1H). Spectral data are in accordance with literature.[22]

(*R*)-*N*-Boc-Bromopropargylglycine methyl ester (39)



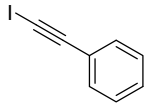
Preparation according to the general procedure B for the preparation of acetylenic bromides afforded **39** (91.4 mg, 0.30 mmol, 69%) as a white solid. R_f = 0.64 (EtOAc/heptanes, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.31 (br d, *J* = 6.8 Hz, 1H), 4.49−4.44 (m, 1H), 3.79 (s, 3H), 2.84−2.69 (m, 2H), 1.46 (s, 9H).

Methyl 3-bromopropiolate (44)



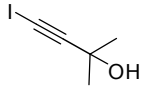
Preparation according to the general procedure B for the preparation of acetylenic bromides, using *N*-iodosuccinimide instead of *N*-bromosuccinimide, afforded **44** (873 mg, 4.66 mmol, 78%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.71 (s, 3H).

Iodophenylacetylene (46)



Preparation according to the general procedure B for the preparation of acetylenic bromides, using *N*-iodosuccinimide instead of *N*-bromosuccinimide, afforded **46** (1.31 g, 5.8 mmol, 96%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.45−7.41 (m, 2H), 7.33−7.28 (m, 3H). Spectral data are in accordance with literature.[23]

3-Iodo-1,1-dimethyl-2-propynol (47)



Preparation similar to the general procedure B for the preparation of acetylenic bromides, using *N*-iodosuccinimide instead of *N*-bromosuccinimide, afforded **47** (232 mg, 0.88 mmol, 88%) as a liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.96 (br s, 1H), 1.53 (s, 6H). Spectral data are in accordance with literature.[24]

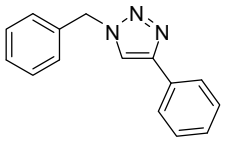
General method C for the preparation of 5-bromo-1,4-disubstituted triazoles.

To a solution of the bromoacetylene (1 equiv) and the azide derivative (1 equiv) in dry THF (0.5 M) was added CuI (5 mol%) and $Cu(OAc)_2$ (5 mol%) The reaction was stirred at 50 °C. The solution was concentrated *in vacuo*, water was added and the product was extracted with DCM (2 ×). The combined organic layers were washed with saturated aqueous

$NaHCO_3$, brine, dried ($MgSO_4$) and evaporated *in vacuo*. The product was purified by flash chromatography using EtOAc/heptane mixtures.

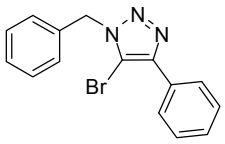
General method D for the preparation of 5-bromo-1,4-disubstituted triazoles.
To a solution of the bromoacetylene (1 equiv) and the azide derivative (1 equiv) in dry THF (0.5 M) was added CuBr (20 mol%) and $Cu(OAc)_2$ (20 mol%) The reaction was stirred at 50 °C. The solution was concentrated, water was added and the product was extracted with DCM (2 ×). The combined organic layers were washed with saturated aqueous $NaHCO_3$, brine, dried ($MgSO_4$) and evaporated *in vacuo*. The product was purified by flash chromatography using EtOAc/heptane mixtures.

1-Benzyl-4-phenyl-[1,2,3]-triazole (9)



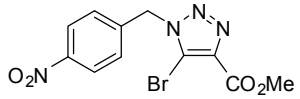
Benzyl azide **7** (30 mg, 0.23 mmol) and phenylbromoacetylene **8** (44 mg, 0.24 mmol) were subjected to the $Cu(OAc)_2$/Na-ascorbate catalyzed [3+2] cycloaddition conditions as described in Chapter 3. Purification by column chromatography afforded **11** (18 mg, 0.08 mmol, 34%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.79 (d, *J* = 7.2 Hz, 2H), 7.66 (s, 1H), 7.44−7.25 (m, 8H), 5.58 (s, 2H). Spectral data are in accordance with literature.[25]

1-Benzyl-5-bromo-4-phenyl-[1,2,3]-triazole (11)



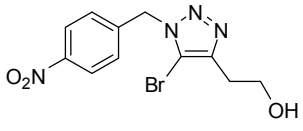
Preparation according to general method C afforded **11** (98 mg, 0.31 mmol, 89%). FTIR (ATR): ν = 3062, 2924, 2850 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.97 (d, *J* = 8.8 Hz, 2H), 7.46−7.34 (m, 8H), 5.63 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 144.8, 133.9, 129.5, 128.9, 128.5, 128.5, 127.7, 126.7, 108.0, 53.2. HRMS (EI) *m/z* calculated for $C_{15}H_{12}Br^{79}N_3$ (M): 313.0215, found: 313.0213.

5-Bromo-4-methylcarboxy-1-(4-nitrobenzyl)-[1,2,3]-triazole (14)



Preparation according to general method C afforded **14** (135 mg, 0.40 mmol, 99%) as a white solid. Preparation according to general procedure D afforded **14** (274 mg, 0.60 mmol, 97%) as a white solid. R_f = 0.38 (EtOAc/heptanes, 1/1). FTIR (ATR): ν = 2954, 1722, 1519, 1342 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.24 (d, *J* = 8.8 Hz, 2H), 7.45 (d, *J* = 8.8 Hz, 2H), 5.73 (s, 2H), 3.99 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 160.1, 148.4, 140.0, 138.2, 128.9, 124.5, 116.6, 52.7, 52.3. HRMS (CI) *m/z* calculated for $C_{11}H_9BrN_4O_4$ (M): 340.9885, found: 340.9887.

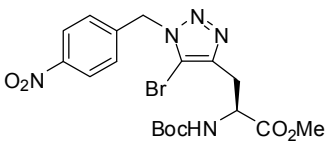
5-Bromo-4-(hydroxyethyl)-1-(4-nitrobenzyl)-[1,2,3]-triazole (19)



Preparation according to general method C afforded **14** (112 mg, 0.34 mmol, 85%) as a white solid. Preparation according to general method D afforded **19** (111 mg, 0.34 mmol, 92%) as a white solid. R_f = 0.22 (EtOAc/heptane, 2/1). FTIR (ATR): ν = 3357, 2848, 1611, 1538, 1517 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.23 (d, *J* = 8.4 Hz, 2H), 7.43 (d, *J* = 8.4 Hz, 2H), 5.65 (s, 2H), 4.01 (q, *J* = 6.0 Hz, 2H), 2.90 (t, *J* = 6.0 Hz, 2H), 2.48 (t, *J* = 6.0 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 148.2, 145.4, 140.9, 128.8, 124.4, 110.2, 61.0, 52.1, 28.3. HRMS (CI)

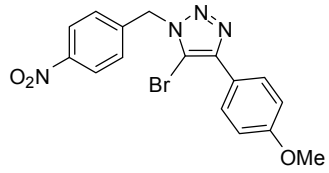
m/z calculated for $C_{11}H_{11}Br^{81}N_4O_3$ (M): 327.9994, found: 327.9993. HRMS (CI): *m/z* calculated for $C_{11}H_{11}Br^{79}N_4O_3$ (M): 326.0014, found 326.0001.

Methyl (*R*)-2-[(*tert*-Butoxycarbonyl)amino]-3-{1-(4-nitrobenzyl)-5-bromo-[1,2,3]-triazol-4-yl}propionate (21)



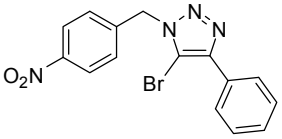
Preparation according to general method C afforded **21** (91 mg, 0.19 mmol, 94%) as a pink oil. Preparation according to general method D afforded **21** (165 mg, 0.34 mmol, 85%) as a pink oil. R_f = 0.45 (EtOAc/heptane, 1/1. FTIR (ATR): ν = 1705, 1528, 1342 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.23 (d, *J* = 8.6 Hz, 2H), 7.38 (d, *J* = 8.4 Hz, 2H), 5.63 (s, 2H), 5.55−5.45 (m, 1H), 4.72−4.60 (m, 1H), 3.73 (s, 3H), 3.19 (bd, *J* = 3.6 Hz, 2H), 1.41 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.5, 155.2, 148.0, 142.9, 140.9, 128.4, 124.2, 111.0, 80.0, 52.6, 52.5, 52.0, 28.3, 28.0. HRMS (ESI) *m/z* calculated for $C_{18}H_{23}^{79}BrN_5O_6$ $(M+H)^+$: 484.0832, found: 484.0810. HRMS (ESI) *m/z* calculated for $C_{18}H_{22}^{79}BrN_5O_6Na$ $(M+Na)^+$: 506.0651, found: 506.0638.

5-Bromo-4-(4-methoxyphenyl)-1-(4-nitrobenzyl)-[1,2,3]-triazole (23)



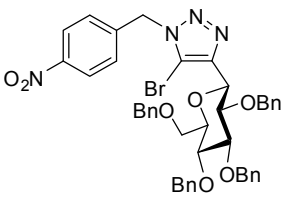
Preparation according to general method C afforded **23** (133 mg, 0.34 mmol, 85%) as an off-white solid. Preparation according to general method D afforded **23** (130 mg, 0.33 mmol, 84%) as an off-white solid. R_f = 0.59 (EtOAc/heptane, 1/1. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.23 (d, *J* = 8.8 Hz, 2 H), 7.90 (d, *J* = 8.8 Hz, 2 H), 7.46 (d, *J* = 8.8 Hz, 2 H), 6.99 (d, *J* = 8.8 Hz, 2 H), 5.71 (s, 2 H), 3.85 (s, 3 H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 160.1, 148.1, 145.3, 141.1, 128.6, 128.2, 124.3, 121.8, 114.2, 107.3, 55.4, 52.1. HRMS (CI) *m/z* calculated for $C_{16}H_{13}Br^{79}N_4O_3$ (M): 388.0171, found: 388.0175. HRMS (CI) *m/z* calculated for $C_{16}H_{13}Br^{81}N_4O_3$ (M): 390.0152, found: 390.0164.

5-Bromo-1-(4-nitrobenzyl)-4-(phenyl)-[1,2,3]-triazole (24)



Preparation according to general method C afforded **24** (134 mg, 0.37 mmol, 93%) as a yellow solid. Preparation according to general method D afforded **24** (57 mg, 0.16 mmol, 39%) as a yellow solid. R_f = 0.50 (EtOAc/heptane, 1/1). ^1H NMR (400 MHz, $CDCl_3$): δ = 8.24 (d, *J* = 8.8 Hz, 2H), 7.99−7.97 (m, 2H), 7.50−7.39 (m, 5H), 5.73 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 150.3, 134.4, 130.3, 129.0, 128.7, 128.6, 127.9, 127.5, 54.5. HRMS (EI) *m/z* calculated for $C_{15}H_{11}Br^{79}N_4O_2$ (M): 358.0065, found: 358.0048.

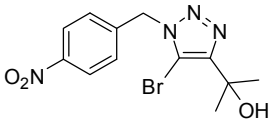
5-Bromo-1-(4-nitrobenzyl)-4-(2,3,4,6-tetra-*O*-acetyl-D-galactopyranosyl)-[1,2,3]-triazole (26)



Preparation according to general method C afforded **26** (54 mg, 0.07 mmol, 63%) as an oil. Preparation according to general method D afforded **26** (87 mg, 0.11 mmol, 98%) as an oil. R_f = 0.70 (EtOAc/heptane, 1/1). FTIR (ATR): ν = 3200, 2103, 1671, 1066 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.07 (d, *J* = 8.6 Hz, 2H), 7.35−7.13 (m, 20H), 6.94 (d, *J* = 6.9 Hz, 2H), 5.57 (q, *J* = 15.7 Hz, 2H), 4.91 (s, 2H), 4.86 (d, *J* = 10.8 Hz, 1H), 4.69 (d, *J* = 11.2 Hz, 1H), 4.63−4.42 (m, 4H), 4.25 (d, *J* = 11.2 Hz, 1H), 4.11 (t, *J* = 9.4 Hz, 1H), 3.82 (t, *J* = 8.9 Hz, 1H), 3.77−3.60 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$):

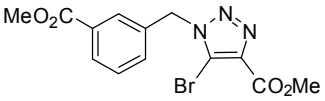
δ = 148,0, 144,3, 140,7, 138,5, 138,1, 138,0, 138,0, 128,5, 128,4, 128,2, 128,0, 127,8, 127,6, 127,6, 127,4, 124,2, 112,0, 86,9, 80,8, 79,8, 78,1, 75,7, 75,2, 74,7, 73,4, 73,2, 69,1, 51,8. HRMS (EI) *m/z* calculated for $C_{43}H_{41}Br^{79}N_4O_7$ (M): 804.2158, found: 804.2147. HRMS (EI) *m/z* calculated for $C_{43}H_{41}Br^{81}N_4O_7$ (M): 806.2138, found: 806.2146.

5-Bromo-4-(2-hydroxy-2-propanyl)-1-(4-nitrobenzyl)-[1,2,3]-triazole (28)



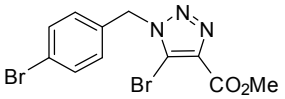
Preparation according to general method D afforded **28** (66 mg, 0.19 mmol, 48%). R_f = 0.55 (EtOAc/heptane, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.21 (d, *J* = 8.8 Hz, 2 H), 7.44 (d, *J* = 8.8 Hz, 2 H), 5.66 (s, 2H), 2,97 (bs, 1H), 1,67 (s, 6H). HRMS (CI) *m/z* calculated for $C_{12}H_{13}Br^{79}N_4O_3$ (M): 340.0171, found: 340.0157. HRMS (CI) *m/z* calculated for $C_{16}H_{13}Br^{81}N_4O_3$ (M): 390.0152, found: 390.0164.

5-Bromo-1-(3-(methoxycarbonyl)benzyl)-4-methylcarboxy-[1,2,3]-triazole (30)



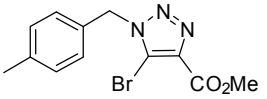
Preparation according to general method C afforded **30** (117 mg, 0.33 mmol, 81%) as a white solid. Preparation according to general method BD afforded **30** (127 mg, 0.36 mmol, 99%) as a white solid. R_f = 0.45 (EtOAc/heptane, 1/1). FTIR (ATR): ν = 2590, 1725, 1531, 1286, 1225 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.06−7.97 (m, 2H), 7.50−7.40 (m, 2H), 5.66 (s, 2H), 3.97 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 166.0, 160.0, 137.7, 133.5, 132.1, 131.0, 130.1, 129.3, 129.0, 116.3, 53.0, 52.6, 52.6 HRMS (EI) *m/z* calculated for $C_{13}H_{13}BrN_3O_4$ $(M+H)^+$: 354.0089, found: 354.0081.

5-Bromo-1-(4-bromobenzyl)-4-methylcarboxy-[1,2,3]-triazole (32)



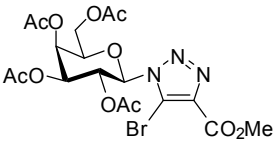
Preparation according to general method C afforded **32** (121 mg, 0.32 mmol, 80%) as a white solid. R_f = 0.51 (EtOAc/heptane, 1/1. FTIR (ATR): ν = 1727, 1524, 1230 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.49 (d, *J* = 8.4 Hz, 3H), 7.17 (d, *J* = 8.4 Hz, 3H), 5.54 (s, 2H), 3.94 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 129.9, 137.7, 132.2, 132.0, 129.5, 13.1, 116.2, 52.7, 52.6. HRMS (EI) *m/z* calculated for $C_{11}H_9Br_2^{79}N_3O_2$ (M): 372.9062, found: 372.9058.

1-(4-Methylbenzyl)-4-methylcarboxy-5-bromo-[1,2,3]-triazole (34)



Preparation according to general method C afforded **34** (100 mg, 0.32 mmol, 81%). R_f = 0.61 (EtOAc/heptane, 1/1. FTIR (ATR): ν = 2946, 1722, 1524, 1450, 1225 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.20−7.14 (m, 4H), 5.57 (s, 2H), 3.97 (s, 3H), 2.33 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 160.1, 138.7, 137.5, 130.1, 129.6, 127.8, 116.1, 53.2, 52.5, 21.5. HRMS (EI) *m/z* calculated for $C_{12}H_{12}BrN_3O_2$ (M): 309.0113, found: 309.0117.

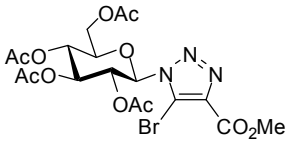
5-Bromo-4-methylcarboxy-1-(2,3,4,6-tetra-*O*-acetyl-D-galactopyranosyl)-[1,2,3]-triazole (36)



Preparation according to general method C afforded **36** (179 mg, 0.33 mmol, 83%). Preparation according to general method D afforded **36** (192 mg, 0.36 mmol, 99%). R_f = 0.30 (EtOAc/heptane, 1/1). FTIR (ATR): ν = 2358, 1744, 1528, 1437, 1368, 1212 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.11 (t, *J* = 9.6 Hz, 1H), 5.84 (d, *J* = 9.4 Hz, 1H), 5.55 (d, *J* =

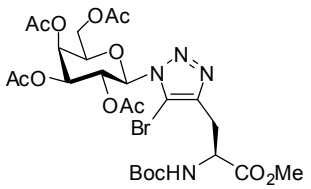
3.3 Hz, 1H), 5.25 (dd, *J* = 10.2, 3.3 Hz, 1H), 3.98 (s, 3H), 4.25−4.15 (m, 3H), 2.22 (s, 3H), 2.05 (s, 3H), 2.02 (s, 3H), 1.88 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.0, 169.9, 169.6, 168.3, 159.7, 137.9, 116.8, 85.5, 73.7, 70.9, 66.7, 66.4, 61.1, 52.2, 20.4, 20.4, 20.3, 20.0. HRMS (CI) *m/z* calculated for $C_{18}H_{23}Br^{79}N_3O_{11}$ $(M+H)^+$: 536.0516, found: 536.0528.

5-Bromo-4-methylcarboxy-1-(2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl)-[1,2,3]-triazole (38)



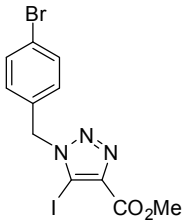
Preparation according to general method C afforded **38** (144 mg, 0.27 mmol, 67%) as a white solid. Preparation according to general method D afforded **38** (172 mg, 0.32 mmol, 91%) as a white solid. R_f = 0.30 (EtOAc/heptane, 1/1). FTIR (ATR): ν = 1748, 1537, 1433, 1368, 1225 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.99 (t, *J* = 9.3 Hz, 1H), 5.86 (d, *J* = 9.5 Hz, 1H), 5.42 (t, *J* = 9.4 Hz, 1H), 5.29 (t, *J* = 9.8 Hz, 1H), 4.26 (dd, *J* = 12.6, 4.8 Hz, 1H), 4.20 (dd, *J* = 12.6, 2.2 Hz, 1H), 4.03−3.99 (m, 1H), 3.98 (s, 3H), 2.08 (s, 6H), 2.04 (s, 3H), 1.87 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.3, 170.0, 169.1, 168.3, 159.7, 138.0, 117.1, 84.7, 74.9, 72.8, 69.0, 67.4, 61.3, 52.4, 20.5, 20.4, 20.1. HRMS (CI) *m/z* calculated for $C_{18}H_{23}Br^{81}N_3O_{11}$ $(M+H)^+$: 538.0496, found: 538.0520.

Methyl (*R*)-2-[(*tert*-Butoxycarbonyl)amino]-3-{1-(2,3,4,6-tetra-*O*-acetyl-D-galactopyranosyl)-5-bromo-[1,2,3]-triazol-4-yl}propionate (43)



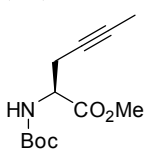
Preparation according to general method C afforded **43** (115 mg, 0.17 mmol, 85%). Preparation according to general method D afforded **43** (89 mg, 0.13 mmol, 65%). R_f = 0.54 (EtOAc/heptane, 2/1). FTIR (ATR): ν = 2971, 1748, 1368 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.01 (t, *J* = 10.0 Hz, 1 H), 5.78 (d, *J* = 9.2 Hz, 1H), 5.58 (d, *J* = 8.0 Hz, 1H), 5.56 (d, *J* = 3.2 Hz, 1H), 5.25 (dd, *J* = 3.2, 10.0 Hz, 1H), 4.69−4.64 (m, 1H), 4.26−4.14 (m, 3H), 3.75 (s, 3H), 2.22 (s, 3H), 2.06 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.4,170.4, 170.1, 170.0, 168.8, 155.3, 143.2, 111.2, 86.0, 79.8, 73.9, 71.1, 66.9, 66.7, 61.3, 52.6, 52.5, 28.4, 27.4, 20.7, 20.6, 20.2. HRMS (FAB) *m/z* calculated for $C_{25}H_{36}BrN_4O_{13}$ $(M+H)^+$: 679.1462, found: 679.1469.

1-(4-Bromobenzyl)-5-iodo-4-methylcarboxy-[1,2,3]-triazole (45)



Preparation similar to general method C, applying an iodoalkyne afforded **45** (82 mg, 0.19 mmol, 99%). R_f = 0.51 (EtOAc/heptane, 1/1). FTIR (ATR): ν =1731. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.49 (d, *J* = 8.6 Hz, 2H), 7.16 (d, *J* = 8.7 Hz, 2H), 5.62 (s, 2H), 3.97 (s, 3H). ^{13}C NMR (75 MHz. $CDCl_3$): δ = 160.4, 142.1, 132.4, 132.1, 129.5, 123.0, 54.1, 53.3. HRMS (EI) *m/z* calculated for $C_{11}H_9Br^{79}IN_3O_2$ (M): 420.8923, found: 420.8924. HRMS (EI) *m/z* calculated for $C_{11}H_9Br^{81}IN_3O_2$ (M): 422.8903, found: 42.8902.

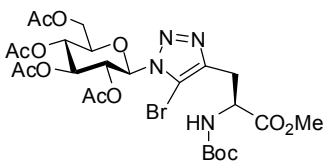
(2*S*)-2-*tert*-Butoxycarbonylamino-4-hexynoic acid methyl ester (40)



(2*S*)-2-Amino-4-hexynoic acid was suspended in DCM (10 ml), Et_3N (0.22 ml, 1.57 mmol) and Boc_2O (243 mg, 1.12 mmol) were added and the reaction mixture was stirred for 3 h. The mixture was concentrated *in vacuo* and purified by chromatography (EtOAc/heptane, 3/1) to afford **31** (121 mg, 0.50 mmol, 68%). R_f = 0.77 (EtOAc/heptane, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.30 (d, *J*

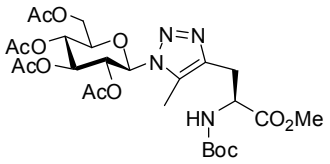
= 7.8 Hz, 1H), 4.43−4.39 (m, 1H), 3.77 (s, 3H), 2.75−2.59 (m, 2H), 1.78 (t, *J* = 2.5 Hz, 3H), 1.46 (s, 9H). Spectral data are in accordance with literature.[19]

3-[5-Bromo-1-(3,4,5-triacetoxy-6-acetoxymethyltetrahydropyran-2-yl)-1*H*-[1,2,3]-triazol-4-yl]-2-*tert*-butoxycarbonylaminopropionic acid methyl ester (41a/42a)



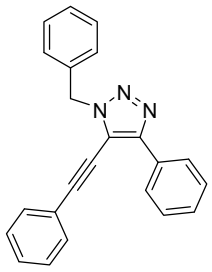
A solution of **30** (20 mg, 0.07 mmol) and **14** (27 mg, 0.07 mmol) in $CDCl_3$ was placed under a pressure of 15 kbar for 2 days at 50°C, resulting in an inseparable mixture of 1,4- and 1,5-regioisomers. Data of mixture of regioisomers: R_f = 0.26 (EtOAc/heptane, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.80 & 5.71 (t, *J* = 9.4 & 9.4 Hz, 2H), 5.84 & 5.44 (d, *J* = 8.2 & 7.6 Hz, 1H), 5.34 & 5.20 (t, *J* = 9.1 & 9.8 Hz, 2H), 4.62−4.48 (m, 1H), 4.32 & 4.29 (d, *J* = 4.0 & 4.0 Hz, 1H), 4.20 (dd, *J* = 12.6, 4.8 Hz, 1H), 4.14 (dd, *J* = 12.6, 2.2 Hz, 1H), 4.04−3.89 (m, 1H), 3.68 & 3.67 (s, 3H), 3.29−3.07 (m, 2H), 2.05 & 2.01 (s , 3H), 2.00 (br s, 3H), 1.98 & 1.97 (s, 3H), 1.81 & 1.79 (s, 3H), 1.37 (br s, 3H). HRMS (ESI) calculated for $C_{25}H_{36}{}^{79}BrN_4O_{13}$ $(M+H)^+$: 679.1462, found: 679.1452. HRMS (ESI) calculated for $C_{25}H_{35}{}^{79}BrN_4O_{13}Na$ $(M+Na)^+$: 701.1282, found: 701.1260.

2-*tert*-Butoxycarbonylamino-3-[5-methyl-1-(3,4,5-triacetoxy-6-acetoxymethyltetrahydro-pyran-2-yl)-1*H*-[1,2,3]-triazol-4-yl]propionic acid methyl ester (41b/42b)



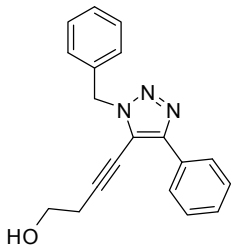
A solution of **31** (17.5 mg, 0.07 mmol) and **14** (26.7 mg, 0.07 mmol) in $CDCl_3$ was placed under a pressure of 15 kbar for 2 days at 50 °C, resulting in an inseparable mixture of 1,4- and 1,5-regioisomers. Data of mixture of regioisomers: R_f = 0.36 (EtOAc/heptanes, 1/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.93 & 5.78 (d, *J* = 10.0 & 9.4 Hz, 1H), 5.68 & 5.50 (t, *J* = 9.3 & 9.5 Hz, 1H), 5.41 (app t, *J* = 9.5 Hz, 1H), 5.35 & 5.23 (t, *J* = 9.6 & 9.7 Hz, 1H), 4.65−4.43 (m, 1H), 4.38 & 4.35 (d, *J* = 3.6 & 3.6 Hz, 1H), 4.28 (dd, *J* = 12.6, 4.9 Hz, 1H), 4.20−4.15 (m, 1H), 4.06−4.02 & 3.98−3.94 (m, 1H), 3.74 & 3.72 (s, 3H), 3.24−3.04 (m, 2H), 2.36 & 2.28 (s, 3H), 2.14 (s, 1H), 2.07 (br s, 4H), 2.06 (s, 1H), 2.04 & 2.02 (s, 3H), 1.86& 1.83 (s, 3H), 1.45 & 1.39 (s, 9H).

1-Benzyl-4-phenyl-5-phenylethynyl-1*H*-1,2,3-triazole (10)



To a solution of 5-bromotriazole **11** (32 mg, 0.10 mmol) in THF (3 mL) were added phenylacetylene **49** (22 µL, 0.42 mmol), $PdCl_2(PPh_3)_2$ (7 mg, 0.01 mmol, 10%), CuI (4 mg, 0.02 mmol) and Et_3N (17 µL, 0.12 mmol) under Schlenck conditions. Next, the mixture was stirred at 70 °C for 5 h and the solvent was removed *in vacuo*. Purification of the crude product by flash column chromatography afforded **11** (27 mg, 0.05 mmol, 46%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.22−8.15 (m, 2H), 7.54−7.31 (m, 13H), 5.68 (s, 2H). HRMS (ESI) calculated for $C_{23}H_{18}N_3$ $(M+H^+)$ 336.1501, found: 336.1480. Spectral data are in accordance with literature.[10]

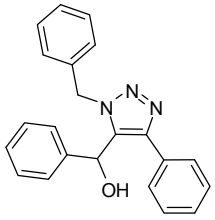
4-(1-Benzyl-4-phenyl-1*H*-1,2,3-triazol-5-yl)but-3-yn-1-ol (50)



To a solution of 5-bromotriazole **11** (63 mg, 0.20 mmol) in THF (3 mL) were added 3-butynol **49** (28 µL, 0.37 mmol), $PdCl_2(PPh_3)_2$ (15 mg, 0.02 mmol), CuI (8 mg, 0.04 mmol) and Et_3N (24 µL, 0.24 mmol) under

Schlenk conditions. Next, the mixture was stirred at 70 °C for 5 h and the solvent was removed *in vacuo*. Purification of the crude product by flash column chromatography afforded **50** (26 mg, 0.09 mmol, 43%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.12−80.9 (m, 2H), 7.47−7.39 (m, 2H), 7.37−7.29 (m, 6H), 5.58 (s, 2H), 3.81 (t, *J* = 6.2 Hz, 2H), 2.78 (t, *J* = 6.2 Hz, 2H).

(1-Benzyl-4-phenyl-1*H*-1,2,3-triazol-5-yl)(phenyl)methanol (51)



To a solution of 5-bromotriazole **11** (31 mg, 0.10 mmol) in THF (3 mL) at −78°C was added Buli (1 equiv) after which stirring was continued for 15 min. Next, a solution of benzaldehyde (13 µL, 0.13 mmol) was added and the mixture was slowly warmed-up to rt. Evaporation of the solvent *in vacuo*, followed by purification of the crude product by flash column chromatography afforded **51** (30 mg, 0.09 mmol, 90%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.63−7.60 (m, 2H), 7.42−7.37 (m, 3H), 7.29−7.27 (m, 3H), 7.24−7.18 (m, 5H), 7.07−7.05 (m, 2H), 6.33 (d, *J* = 5.1 Hz, 1H), 5.49 (d, *J* = 15.1 Hz, 1H), 5.27 (d, *J* = 15.1 Hz, 1H).

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The great tragedy of science-
The slaying of a beautiful hypothesis,
by an ugly fact.

-Thomas Huxley

A tidy fumehood means a lazy chemist.

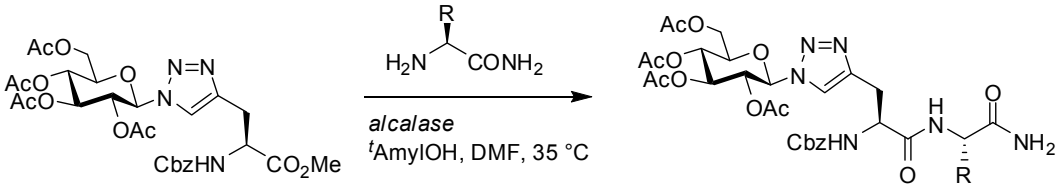
-Jöns Jacob Berzelius

Enzymatic Hydrolysis and Coupling of Triazolyl Amino Acids

CHAPTER 6

Abstract*:

Chemoenzymatic peptide coupling of glycosylated triazolyl- and amide-linked amino acid methyl esters was effected by *alcalase*, a proteolytic enzyme mixture produced by *Bacillus licheniformis*. Both the common amide-linked as well as the unnatural triazole-linked substrates were ligated to amino acid amides in moderate to good yields, producing dipeptides. Moreover, a first study towards the enzymatic glycosylation of triazole-linked glycopeptides is presented.



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6.1 INTRODUCTION

Peptides are generally defined as oligomers or polymers of amino acids with less than 100 monomers. As such, peptides are essential molecules in the fields of healthcare and nutrition, for example as food additives[1] and therapeutics.[2] Consequently, over the years a variety of different technologies have been developed for the efficient production of pure peptides, including extraction from natural sources,[3] production *via* recombinant DNA technology,[4] expression in cell-free systems,[5] production in genetically modified animals[6] or plants, solution based chemical synthesis,[7] resin based chemical synthesis[8] and enzymatic ligation technology.[9] Natural peptides can be readily obtained by most of these techniques, but for designed or non-natural peptides only chemical and chemoenzymatic syntheses can be applied. From these two strategies, the chemical procedure clearly stands out as the most mature technology. Nevertheless, lack of specificity and environmental burden of chemical synthesis, has led to an increasing interest in chemoenzymatic biocatalysis over the last decade.[10] Chemoenzymatic synthesis is characterized by a number of specific advantages over chemical peptide synthesis: (a) chemoenzymatic peptide coupling reactions are free from racemization, (b) side-chain protection can mostly be omitted, (c) the coupling reagent (enzyme) can often be recycled, (d) reactions proceed under mild conditions, (e) conditions are environmentally friendly and (f) couplings proceed with high specificity.

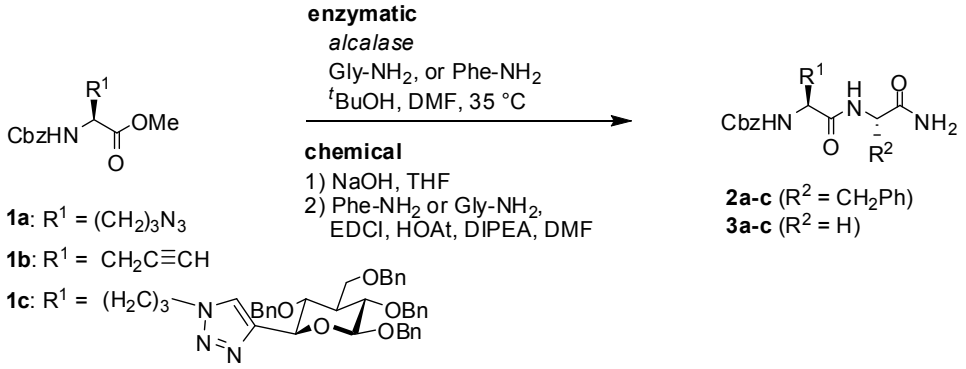
Two distinguishable strategies can be identified for chemoenzymatic ligation, a thermodynamically (or equilibrium) controlled approach and a kinetically controlled variant. In the *thermodynamic* approach, involving the reversal of proteolysis, an *N*-protected amino acid (bearing a free carboxyl group) is condensed with a *C*-protected amino acid. Since the reaction is driven by equilibrium, substrate solubility must be high, while solubility of the product must be low to shift the balance to the product side (Le Chatelier's principle). Typically, equilibrium-controlled synthesis is pH-sensitive, and the acidity of the reaction must be chosen carefully, since only the non-ionic forms of both acid and amine are involved in the reaction.[11] In contrast, a *kinetically* controlled approach depends on the use of activated carboxyl compounds. The advantage is that much less enzyme is required, the reaction time to obtain maximum product yield is significantly shorter, and the reaction can be carried out between pH 7 and 9. Another important advantage is the fact that the yield is predominantly determined by the properties of the enzyme,

however, a drawback of kinetic control is the fact that in an aqueous medium 100% product formation is nearly never attained, due to increasing hydrolysis of the newly formed peptide bond in the product formed. Suitable enzymes for *kinetic* peptide synthesis include commercially available serine, metallo-, and aspartate proteases.[12]

Moreover, it was demonstrated by Chen *et al.* that *alcalase,* a cheap and commercially available proteolytic enzyme mixture produced by *Bacillus licheniformis*, containing subtilisin Carlsberg as the major enzyme component, can be conveniently applied for enzymatic peptide bond formation as well.[12,13] In addition, *alcalase* was found to be stable in a variety of organic solvents, retaining more than 65% of the original activity in *tert*-amyl alcohol after 10 days, and more than 60% in *tert*-butyl alcohol after 6 days. Based on these results, we set out to investigate the chemoenzymatic synthesis of dipeptides *via C*-terminal elongation of propargylglycine, azidopropylglycine, and glycosylated triazolyl-containing amino acid methyl esters ('activated donor') with a proteinogenic amino acid amide ('inactive acceptor').[14] In order to make a fair comparison, both conventional solution-phase chemical peptide synthesis as well as an enzymatic procedure under the action of the protease *alcalase* were applied, results of which are summarized in Table 1.

To this end, three Cbz-protected amino acid methyl esters (ornitine-derived (3-azidopropyl)glycine **1a**, propargylglycine **1b**, and glycosyl triazolylmethylglycine **1c**) were coupled to an amino acid amide (phenylalanine amide or glycine amide) by two routes: (a) under the action of *alcalase* or (b) by basic ester hydrolysis followed by carbodiimide-mediated coupling. Chemoenzymatic coupling was executed by coevaporation of the methyl ester with DMF, followed by dissolution of both methyl ester and amino acid amide in *tert*-amyl alcohol. Subsequently, *alcalase* was repeatedly added, until HPLC analysis indicated complete conversion of starting material. Much to our delight, *alcalase*-mediated coupling proceeded smoothly for both protected (*S*)-azidoornithine (entries 1 and 4) and (*S*)-propargylglycine (entries 2 and 5) with either phenylalanine or glycine amide, providing the desired dipeptides in excellent yield (entries 1-2 and 4-5).

Table 1. *Initial enzymatic synthesis of dipeptide derivatives.*



entry	substrates	nucleophile	chemical yield (%)	enzymatic yield (%)	dipeptide
1	**1a**	Phe-NH_2	69	77	**2a**
2	**1b**		55	79	**2b**
3	**1c**		86	40[a]	**2c**
4	**1a**	Gly-NH_2	80	93	**3a**
5	**1b**		60	80	**3b**
6	**1c**		82	10	**3c**

[a]Conversion of the acyl donor as determined by HPLC

Interestingly, the chemoenzymatic coupling reaction proceeded with considerably higher yields as compared to the chemical peptide ligation, although it has to be noted that one equivalent of amide was added in the chemical synthesis, in contrast to 4 equivalents in the enzymatic ligation. On the other hand, for the triazole-linked glycosylated amino acid **1c** the chemical coupling procedure was preferable since chemoenzymatic coupling failed to afford the desired products **2c** and **3c** in good yields (entries 3 and 6). In fact, HPLC analysis showed only 40% conversion after 8 days, resulting in an isolated yield of approximately 10%.

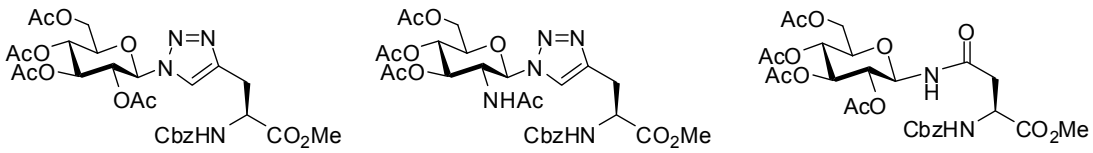


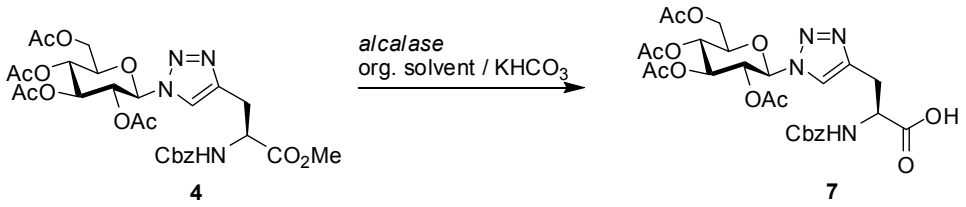
Figure 1. *Substrates for enzymatic ligation.*

Nevertheless, the findings that glycosylated triazole-linked substrates appeared to be suitable for enzymatic ligation was taken as an encouragement, since it was anticipated that the yield could be raised using less sterically hindered and better soluble substrates as depicted in Figure 1. Moreover, we studied possible differences in chemoenzymatic ligation between triazole-linked glycosidic amino acids **4** and **5** and the naturally occurring amide-linked glycoamino acid **6**.

6.2 HYDROLYSIS STUDIES

In order to validate the hypothesis that acetylated sugars are better substrates for *alcalase* than the bulky benzylated compound **1c**, some hydrolysis experiments were conducted with substrate **4** before coupling reactions were attempted (Table 2). Therefore, compound **4** was dissolved in a 1:1 mixture of *tert*-butyl or *tert*-amyl alcohol and aqueous potassium bicarbonate, and treated with *alcalase*. The reaction in either *tert*-butyl or *tert*-amyl alcohol proceeded equally fast, and we were delighted to find that despite the fact that compound **4** did not dissolve completely, after 24 hours more than 80% of the substrate was hydrolyzed by the action of *alcalase* (entries 1 and 2).

Table 2. *Results of hydrolysis experiments of compound* **4** *under the action of alcalase.*



4 **7**

		Acid (**7**) (%)[a] at t (h)			
entry		t = 0.5	t = 4	t = 6	t = 24
1	^{t}BuOH / 1 M aq. $KHCO_3$ (*v/v* = 1/1)	5	-	32	81
2	^{t}amylOH / 1 M aq. $KHCO_3$ (*v/v* = 1/1)	3	22	-	82
3	1M aq. $KHCO_3$ (w/o *alcalase*)	<1.4[b]	<1.4[b]	-	<1.4[b]
4	^{t}BuOH / 1M aq. $KHCO_3$ / DMF (*v/v* = 10/9/1)	4	17	-	59
5	^{t}BuOH / 1M $KHCO_3$ / acetonitrile (*v/v* = 10/9/1)	4	21	-	66

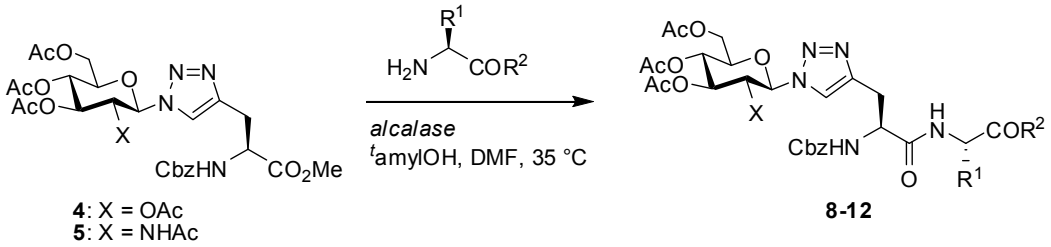
[a]Yield as determined by HPLC [b]detection limit.

To enhance solubility, addition of DMF or acetonitrile in combination with water was applied in other systems,[15] although *alcalase* has been reported to be insoluble in acetonitrile. Unfortunately, in our hands upon addition of DMF (entry 4) or acetonitrile (entry 5), the rate of hydrolysis rapidly declined. In addition, a control experiment was performed without the addition of *alcalase* (entry 3) to exclude that ester hydrolysis was due to the slightly alkaline conditions. Nevertheless, 80% hydrolysis after 24 hours was taken as a promising result compared to the extremely slow reaction with the benzylated substrate **1c** reported earlier.

6.3 ENZYMATIC LIGATION OF TRIAZOLE-LINKED GLYCOSIDIC AMINO ACIDS

Both *tert*-butyl and *tert*-amyl alcohol showed similar results in the hydrolysis studies. However, *tert*-amyl alcohol was preferred as solvent for the coupling experiments, since *alcalase* is reported to be more stable in *tert*-amyl alcohol.[12] Unless stated otherwise, dried *alcalase* was added three times a week during the course of the reaction in all subsequent experiments.

Table 3. *Application of triazole-linked glycosidic substrates in enzymatic coupling experiments.*



4: X = OAc
5: X = NHAc

8-12

entry	substrate	R^1	R^2	t (days)	product	HPLC yield[c]	isolated yield
1	**4**	H	NH_2	6	**8**	100%[a,b]	98%
2	**4**	Bn	NH_2	8	**9**	57%[a] (34%)[b]	30%
3	**4**	Bn	Val-NH_2	15	**10**	53%[a]	n.d.
4	**5**	H	NH_2	11	**11**	31%[a,d]	n.d.
5	**5**	Bn	NH_2	21	**12**	73%[a,d]	n.d.

[a]Optimal HPLC yield. [b]HPLC yield before quenching and isolation. [c]The given HPLC values contain an error of approximately 8% caused by weighing the reactants, pipetting the enzyme, pipetting the thick reaction mixtures for taking HPLC samples and the HPLC instrument itself. [d]*Alcalase* was added two times a week.

First, chemoenzymatic ligation was tested between *N*-triazole-linked glycosidic amino acid (^{N}TGA) **4** and glycine amide (Table 3, entry 1). We were delighted to find that the desired dipeptide **8** was formed smoothly and could be isolated in nearly quantitative yield after 6 days (entry 1). A less satisfactory result was obtained when the same methyl ester **4** was condensed with phenylalanine amide as a nucleophile, resulting in the isolation of dipeptide **9** in a rather poor 30% yield along with several byproducts. Unfortunately, the identity of the impurities could not be established, although it was clear that enzymatic hydrolysis of the starting material was not an issue. Since glycine amide was added in excess to the reaction mixture, formation of a tripeptide (^{N}TGA-Gly-Gly-NH_2) might be one of the possibilities.

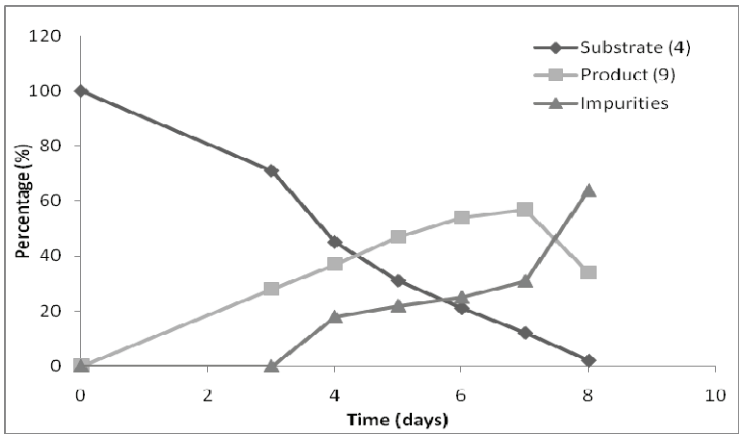
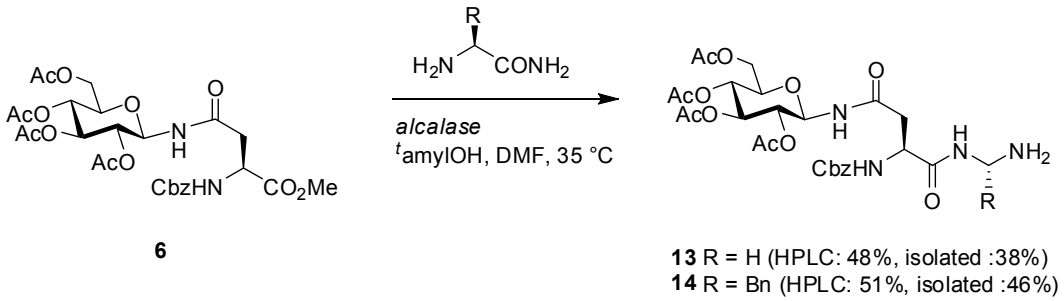


Figure 2. *Reaction profile of triazole-linked glycosidic amino acid* **4** *with glycine amide.*

A closer inspection of the reaction profile with HPLC, however, showed that at first the reaction advanced slowly leading to a maximal yield of 57% after 7 days, along with 30% of unidentified side-products (Figure 2). Only after this time, the formation of side-products increased rapidly with concomitant decrease in yield of dipeptide **9** to 34% as judged by HPLC, resulting in the 30% isolated yield (entry 2). Next, a slightly more challenging ligation was carried out, involving addition of the dipeptide nucleophile H-Phe-Val-NH_2 to methyl ester **4** in order to prepare tripeptide **10** (entry 3). It was rewarding to find that *alcalase*-mediated ligation led to an 53% yield (HPLC) of tripeptide **10**, thereby clearly showing that the chemoenzymatic coupling is not restricted to the formation of dipeptides. Next, the fully acetylated glucosamine-containing triazolylamino acid **5** was utilized as acyl donor. Interestingly, contrasting results were encountered with respect to condensation of glycine amide (entry 4) and phenylalanine amide (entry 5) as

compared to the glucose-containing donor **4**. Thus, an HPLC yield of 73% of **12** was found employing phenylalanine amide (entry 5), whereas a poor 31% of **11** was obtained when glycine amide was applied as the nucleophile (entry 4). Although some solubility problems were encountered during the latter enzymatic ligations, currently we have no satisfactory explanation for the unexpected reversal in yield between substrates **4** and **5**.

Finally, a condensation of the naturally occurring amide-linked glycosidic amino acid **6** with glycine and phenylalanine amide was performed under identical conditions in the presence of *alcalase*, as depicted in Scheme 1. In case of this particular substrate, both nucleophiles reacted similarly, reaching an optimum yield of approximately 50% (HPLC) for both dipeptides **13** and **14**. The respective dipeptides were eventually isolated in yields somewhat lower than the indicated HPLC values.

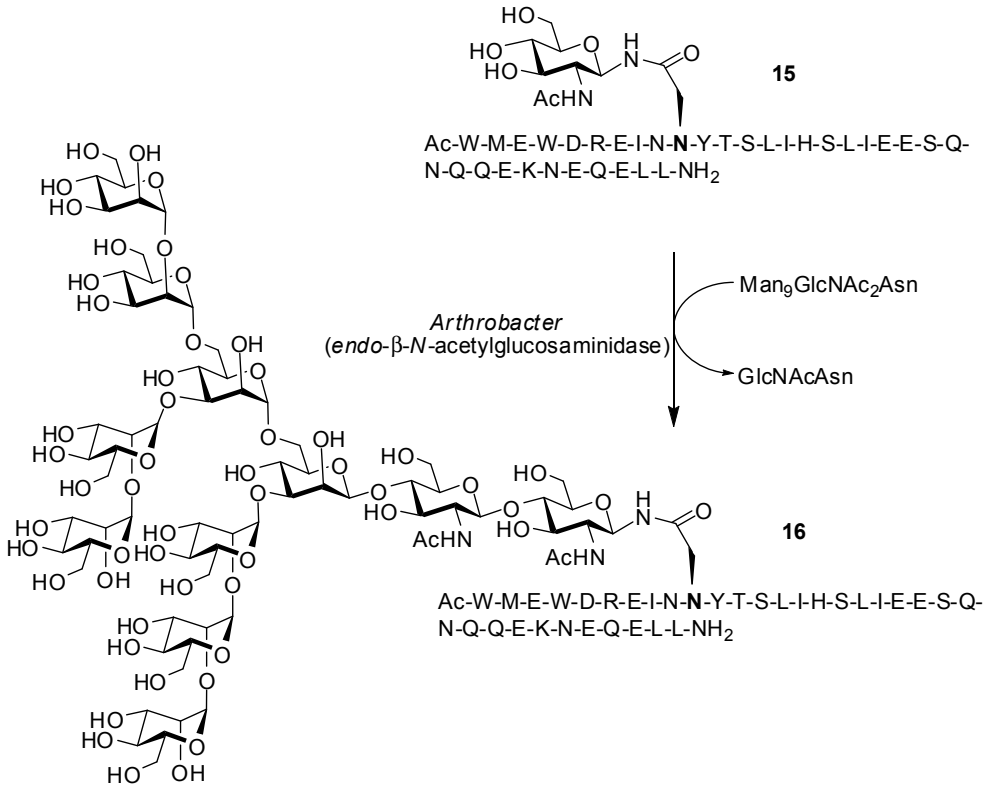


Scheme 1. *Application of natural amide-linked glycosidic substrates in enzymatic coupling experiments.*

6.4 CHEMOENZYMATIC GLYCOSYLATION

Having successfully achieved the chemoenzymatic preparation of glycosylated triazole-linked di- and tripeptides, we anticipated that the isosteric triazolylpeptides themselves could serve as substrates for further chemoenzymatic functionalization, in particular by glycosylation. In this respect, an interesting chemoenzymatic procedure was recently described by Xi *et al.*,[16] involving the glycosylation of the naturally occurring 34-mer peptide **15**. Thus, using *arthrobacter* as key enzyme, undecaglycopeptide **16** was prepared by transglycosylation of glycosylated peptide **15** as depicted in Scheme 2.

In order to evaluate the behavior of triazole-linked glycosidic amino acids several of the derivatives prepared earlier (described in Chapter 3) were deprotected to obtain the three model substrates **17-19** as well as the C34 glycopeptide **20** depicted in Figure 3.



Scheme 2. *Chemoenzymatic synthesis of glycopeptides by Xi et al.*[16]

Currently, compounds **17-20** are under evaluation as transglycosylation acceptors in the group of Prof. Lai-Xi Wang (University of Maryland, Maryland, USA). Conclusive results cannot be presented at this point, but preliminary experiments conducted with the triazole-linked C34-mer have shown that *arthrobacter* is indeed capable of recognizing the substrate so that subsequent glycosylation will provide the triazole-linked analogue of **16**.

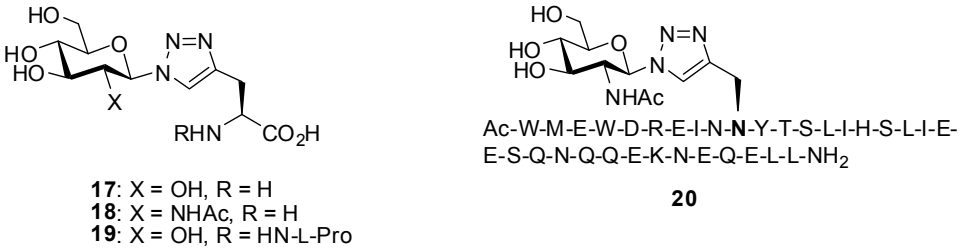


Figure 3. *Deprotected triazole-linked glycoamino acids and peptides.*

Unfortunately, substrates **17-19** have not been tested yet. Nevertheless, based on the successful glycosylation of **20**, it is safe to conclude that triazoles appear to function as useful amide isosteres in biological active systems.

6.5 CONCLUSION

Proof of principle was delivered for the use of triazole-linked glycosyl amino acids as acyl donors in the chemoenzymatic ligation with amino acid derivatives and peptides as nucleophile. Although coupling reactions proceeded relatively slowly, presumably due to low solubility of the substrates and/or steric factors, triazole-linked glucose or glucosamine were both successfully incorporated into dipeptides, similar to the amide-linked glycoamino acids, under the action of the proteolytic enzyme mixture *alcalase*. Conditions may be further optimized to efficiently apply *alcalase* for chemoenzymatic peptide synthesis, including the incorporation of triazole-linked glycosidic amino acids. Moreover, a preliminary study towards the enzymatic glycosylation of triazole-linked glycopeptides was presented, showing that a monoglycosylated 34-mer peptide could be effectively transglycosylated under the action of *arthrobacter* as key enzyme.

6.6 ACKNOWLEDGMENT

C. Hawner, H. C. P. F. Roelen, and R. W. Wiertz (DSM Pharmaceutical Products, Geleen, The Netherlands) are gratefully acknowledged for their contributions to this chapter. Dr. P. J. L. M. Quaedflieg (DSM Pharmaceutical Products, Geleen, The Netherlands) is acknowledged for the helpful discussions and advice on the enzymatic reactions.

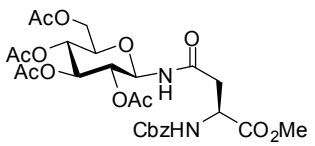
6.7 EXPERIMENTAL

Alcalase type DX PLN04842 was purchased from Novozymes (Bagsvaerd, Denmark) as a brown liquid with a specific activity of 2.5 U/mL. For the hydrolysis experiments this enzyme solution was used as such. For the (anhydrous) peptide coupling reactions the water in the *alcalase* solution was removed using the method of Chen *et al*.[12] As a matter of fact, the aqueous *alcalase* (0.25 mL) and absolute ethanol (0.5 mL) were mixed in an Eppendorf cup and the resulting suspension was agitated on a Vortex mixer for 5 min and centrifuged (3000 rpm) for 10 min to spin down the enzyme. The supernatant was decanted and the enzyme resuspended in absolute EtOH (0.5 mL), agitated for 5 min on a Vortex mixer and spun down by centrifugation. This procedure was repeated once with absolute EtOH (0.5 mL) and finally once with dry ^{t}amylOH (0.5 mL). The resulting enzyme was resuspended in ^{t}amylOH (1.0 mL) for use in the enzymatic peptide coupling reactions. All other commercially available reagents were used as received. HPLC analyses were performed with an Inertsil RP-18 column (25 × 0.46 cm, RP-18, 5 μm).

General procedure for chemoenzymatic peptide coupling:
The *N*-Cbz-protected amino acid methyl ester (0.39 mmol) was dried *via* coevaporation with DMF (2 × 5 mL) and dissolved in ^{t}amylOH (4.0 mL). After adding a solution of Phe-NH_2 or Gly-NH_2 (1.5 mmol) in ^{t}amylOH (2.0 mL), the reaction mixture was stirred at 37 °C. Subsequently, the dried *alcalase* suspension in *tert*-amyl alcohol (1 mL) was added and the reaction mixture was stirred at 37 °C. Samples were taken at regular time intervals and analyzed by HPLC. **Workup**: After almost complete conversion of **1**, the reaction mixture was concentrated *in vacuo* to remove most of the volatiles. The residue was taken up in EtOAc (50 mL) and H_2O (20 mL), to which a few drops of 1 N aqueous HCl were added. The aqueous layer was extracted with EtOAc (3 × 30 mL) and the combined organic phase was washed with aqueous $KHCO_3$ (1 M, 40 mL), aqueous HCl (1 N, 40 mL) and brine (40 mL), dried (Na_2SO_4) and concentrated *in vacuo*. Analytically pure samples were obtained by recrystallization. A new load of enzyme (dried precipitate from 500 μL of enzyme solution) was added 3 times a week. **Quenching solution for HPLC samples**: 50% KH_2PO_4 (0.1 M, pH 5), 50% MeCN.

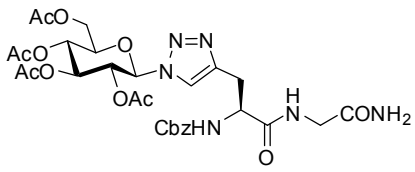
Hydrolysis experiments
To a suspension of methyl (*R*)–2–[(benzyloxycarbonyl)amino]–3–{1–(2,3,4,6–tetra–*O*–acetyl–β–D–gluco pyranosyl)[1,2,3]triazol–4–yl}propionate (95 mg, 0.15 mmol) in 1 M $KHCO_3$ (450 μL) was added the organic solvent (mixture) (450 μL) and *alcalase* solution (43 μL). Next, the reaction mixture was stirred at room temperature. Samples were taken at regular time intervals and analyzed by HPLC. **Quenching solution for HPLC samples**: 50% KH_2PO_4 (0.1 M, pH 5), 50% MeCN.

Cbz-(*S*)-(*N*-1-Glu(Ac)$_4$-Asn)-OMe (6)



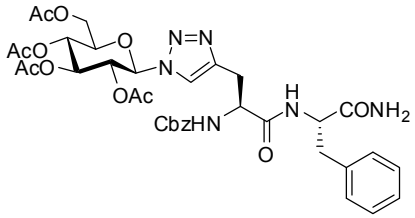
The amide-linked sugar amino acid was prepared from glucopyranosyl amine and Cbz-Asp-O^{t}Bu *via* a procedure reported by van Ameijde *et al.*[17] involving peptide coupling catalyzed by BOP followed by deprotection using TFA/DCM (4 h, rt) to afford the *N*-glycosylasparagine **6** (550 mg, 0.9 mmol, 73%) as a white solid . ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.38−7.34 (m, 5H), 6.38 (d, *J* = 9.2 Hz, 1H), 6.01−5.92 (m, 1H), 5.29 (t, *J* = 9.5 Hz, 1H), 5.21 (t, *J* = 9.3 Hz, 1H), 5.09−5.02 (m, 1H), 4.90 (t, *J* = 9.6 Hz, 1H), 4.66−4.57 (m, 1H), 4.29 (dd, *J* = 12.5 Hz, 1H), 4.07 (dd, *J* = 12.5 Hz, 1H), 3.78 (ddd, *J* = 10.4, 2.2 Hz, 1H), 2.93−2.84 (m, 1H), 2.71 (dd, *J* = 16.3 Hz, 1H), 2.03 (s, 3H), 2.02 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 3.73 (s, 3H), 5.12 (s, 2H). Spectral data are in accordance with literature.[18]

Cbz-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-Gly-NH$_2$ (8)



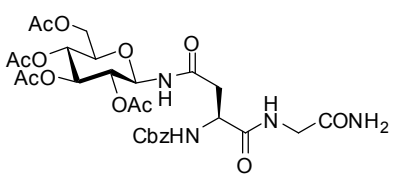
Preparation according to the general procedure for the peptide coupling afforded **8** (260 mg, 0.38 mmol, 98%) as a white solid. R_f (EtOAc) = 0.52. IR (KBr) ν = 3292, 2948, 1753, 1687, 1535 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$/MeOD): δ = 7.93 (s, 1 H), 7.42−7.25 (m, 5 H), 5.99−5.90 (m, 1 H), 5.53 (s, 1 H), 5.50−5.45 (m, 2 H), 5.34−5.23 (m, 1 H), 5.11 (d, *J* = 12.4 Hz, 1 H), 5.08 (d, *J* = 12.4 Hz, 1 H), 4.49 (t, *J* = 6.2, 6.2 Hz, 1 H), 4.32 (dd, *J* = 12.6, 4.8 Hz, 1 H), 4.18 (dd, *J* = 12.6, 2.1 Hz, 1 H), 4.16−4.10 (m, 1 H), 3.92 (d, *J* = 17.1 Hz, 1 H), 3.84 (d, *J* = 17.0 Hz, 1 H), 3.29−3.18 (m, 2 H), 2.09 (s, 1 H), 2.08 (s, 1 H), 2.04 (s, 1 H), 1.83 (s, 1 H). ^{13}C NMR (75 MHz, $CDCl_3$/MeOD): δ = 172.1, 171.5, 170.5, 169.7, 169.3, 168.9, 156.3, 135.6, 127.9, 127.6, 127.4, 121.6, 85.0, 74.2, 72.1, 70.2, 67.3, 66.6, 61.1, 54.1, 41.8, 27.1, 19.7, 19.6, 19.1. HRMS (ESI) *m/z* calculated for $C_{29}H_{36}N_6O_{13}Na$ $(M+Na)^+$: 699.2238, found: 699.2213.

Cbz-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-L-Phe-NH$_2$ (9)



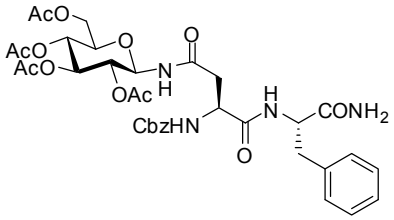
Preparation based on the general procedure for the peptide coupling using 1.00 g (1.58 mmol) of **4** afforded **9** (300 mg, 0.46 mmol, 30%) as a white solid. R_f (EtOAc) = 0.60. IR (KBr) ν = 3351, 2942, 1748, 1693, 1666, 1524 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$/MeOD): δ = 7.74 (s, 1 H), 7.41−7.16 (m, 10 H), 5.96−5.87 (m, 1 H), 5.53 (s, 1 H), 5.49−5.41 (m, 2 H), 5.06 (s, 2 H), 4.63 (dd, *J* = 8.1, 5.9 Hz, 1 H), 4.42 (t, *J* = 6.4, 6.4 Hz, 1 H), 4.31 (dd, *J* = 12.6, 4.9 Hz, 1 H), 4.16 (dd, *J* = 12.6, 2.1 Hz, 1 H), 4.11 (ddd, *J* = 10.2, 4.8, 2.1 Hz, 1 H), 3.20−3.15 (m, 2 H), 3.05 (dd, *J* = 15.16, 6.86 Hz, 1 H), 2.96 (dd, *J* = 13.90, 8.27 Hz, 1 H), 2.09 (s, 3 H), 2.06 (s, 3 H), 2.04 (s, 3 H), 1.82 (s, 3 H). ^{13}C NMR (75 MHz, $CDCl_3$/MeOD): δ = 173.6, 170.7, 170.5, 169.8, 169.4, 168.9, 156.1, 143.1, 136.2, 135.6, 128.6, 128.5, 128.0, 127.7, 127.4, 126.3, 121.4, 85.0, 74.3, 72.2, 70.1, 67.3, 66.6, 61.2, 53.9, 53.6, 36.8, 27.2, 19.8, 19.7, 19.2. HRMS (ESI) *m/z* calculated for $C_{36}H_{42}N_6O_{13}Na$ $(M+Na)^+$: 789.2708, found 789.2675.

Cbz-(*S*)-(*N*-1-Glu(Ac)$_4$-Asn)-Gly-NH$_2$ (13)



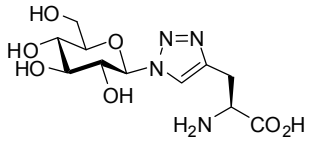
Preparation according to the general procedure for the peptide coupling afforded **13** (98 mg, 0.15 mmol, 38%) as a white solid. FTIR (ATR): ν = 3317, 1752, 1671 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.37 (bs, 5H), 7.04 (m, 1H), 6.65 (d, *J* = 9.0 Hz, 1H), 6.37 (s, 1H), 6.10 (d, *J* = 7.4 Hz, 1H), 5.46 (s, 1H), 5.31 (dd, *J* = 11.4 Hz, 2H), 5.16 (t, *J* = 12.0 Hz, 1H), 5.05 (t, *J* = 9.7 Hz, 1H), 4.90 (t, *J* = 9.6 Hz, 1H), 4.63−4.52 (m, 1H), 4.27 (dd, *J* = 12.5 Hz, 1H), 4.18−4.02 (m, 2H), 3.84−3.69 (m, 2H), 3.02 (dd, *J* = 16.4 Hz, 1H), 2.91 (d, *J* = 10.6 Hz, 1H), 2.62 (dd, *J* = 16.4 Hz, 1H), 2.02 (s, 3H), 2.01 (s, 3H), 2.08 (s, 3H), 2.07 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.5, 171.0, 171.0, 170.8, 170.1, 169.5, 169.0, 155.7, 135.2, 128.2, 128.0, 127.8, 77.5, 76.7, 73.2, 72.0, 69.9, 67.5, 67.2, 61.1, 51.0, 42.5, 20.2, 20.2, 20.1. HRMS (ESI) *m/z* calculated for $C_{28}H_{36}N_4O_{13}Na$ (M+Na)$^+$: 675.2126, found 675.2116.

Cbz-(*S*)-(*N*-1-Glu(Ac)$_4$-Asn)- L-Phe-NH$_2$ (14)



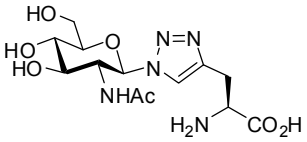
Preparation according to the general procedure for the peptide coupling afforded **14** (135 mg, 0.18 mmol, 46%) as a white solid. FTIR (ATR): ν = 3399, 1753, 1631 cm^{-1}. ^{1}H NMR (400 MHz, D_2O): δ = 7.43−7.16 (m, 10H), 6.88 (d, *J* = 7.9 Hz, 1H), 6.72 (d, *J* = 9.3 Hz, 1H), 6.16 (d, *J* = 7.9 Hz, 1H), 6.00 (s, 1H), 5.40 (s, 1H), 5.29 (t, *J* = 9.6 Hz, 1H), 5.16 (t, *J* = 9.2 Hz, 1H), 5.09−5.01 (m, 2H), 4.91 (t, *J* = 9.6 Hz, 1H), 4.60−4.51 (m, 1H), 4.50−4.40 (m, 1H), 4.26 (dd, *J* = 12.6 Hz, 1H), 4.16−4.07 (m, 2H), 3.78 (ddd, *J* = 10.1, 2.0 Hz, 1H), 3.17−3.01 (m, 2H), 2.76 (dd, *J* = 16.1 Hz, 1H), 2.59 (dd, *J* = 16.1 Hz, 1H), 2.07 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.7, 172.0, 171.2, 170.4, 196.7, 169.6, 169.4, 146.5, 136.0, 127.6, 127.3, 127.2, 76.9, 72.9, 72.9, 70.1, 67.7, 66.2, 61.3, 51.2, 41.6, 36.5, 29.0, 18.7, 18.7. HRMS (ESI) *m/z* calculated for $C_{35}H_{42}N_4O_{14}Na$ (M+Na)$^+$: 765.2595, found: 765.2605.

(*S*)-T4M(1-[β-D-Glc])-OH (18)



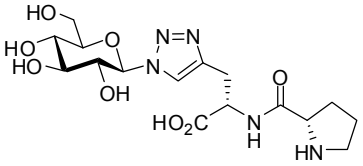
Methyl (*R*)-2-[(benzyloxycarbonyl) amino]-3-{1-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl) [1,2,3]triazol-4-yl} propionate (**43**, Chapter 3) (100 mg, 0.16 mmol) was dissolved in MeOH (3 mL), Pd/C (2 mg, 12 mol%) was added and the reaction was stirred for 45 min under na atmosphere of H_2. Next, the suspension was filtrated, a catalytic amount of K_2CO_3 was added and the mixture was stirred overnight. Purification of the product with a basic ion exchange column (IRA-410) afforded the triazole-linked glycosidic amino acid **18** (40 mg, 80%) as a white solid. FTIR (ATR): ν = 3309, 2362, 2327, 1623 cm^{-1}. ^{1}H NMR (400 MHz, D_2O): δ = 7.98 (s, 1H), 5.61 (d, *J* = 9.2 Hz, 1H), 3.98 (t, *J* = 5.8 Hz, 1H), 3.86 (t, *J* = 9.2 Hz, 1H), 3.76 (d, *J* = 11.0 Hz, 1H), 3.66−3.55 (m, 3H), 3.48 (t, *J* = 9.2 Hz, 1H), 3.31−3.17 (m, 2H). ^{13}C NMR (75 MHz, D_2O): δ = 172.3, 141.7, 123.3, 86.9, 78.4, 75.4, 71.7, 68.4, 59.9, 53.7, 25.8. HRMS (ESI) *m/z* calculated for $C_{11}H_{18}N_4O_7Na$ (M+Na)$^+$: 341.1073, found: 341.1081.

(*S*)-T4M(1-[β-D-GlcNAc])-OH (19)



A solution of 3-{1-[2-(Acetylamino)-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-glucopyranosyl] [1,2,3] triazol-4-yl}-(R)-2- [(tert-butoxycarbonyl) amino]propanoic acid (**63**, Chapter 3) (100 mg, 0.17 mmol) in HCl/EtOAc (2 mL) was stirred for 30 min. Next, the solvent was evaporated *in vacuo* and the crude product was redissolved in MeOH (3 mL), a catalytic amount of K_2CO_3 was added and the mixture was stirred overnight. Purification of the product with an acidic ion exchange column (IRA-120) afforded the triazole-linked glycosidic amino acid **19** (30 mg, 0.08 mmol, 49%) as a white solid. ^{1}H NMR (400 MHz, D_2O): δ = 8.02 (s, 1H), 5.69 (d, *J* = 9.7 Hz, 1H), 4.26 (t, *J* = 6.1 Hz, 1H), 4.11 (t, *J* = 9.9 Hz, 1H), 3.79 (dd, *J* = 12.3 Hz, 1H), 3.71−3.49 (m, 5H), 3.29 (d, *J* = 6.1 Hz, 2H), 2.07 (s, 3H). ^{13}C NMR (75 MHz, D_2O): δ = 173.7, 170.2, 140.7, 123.0, 85.9, 78.4, 73.0, 68.8, 59.9, 54.7, 51.9, 25.1, 21.1. LRMS (ESI) *m/z* calculated for $C_{13}H_{21}N_5O_7$ $(M-H)^-$: 359.14, found: 358.2.

(*S*)-Pro-(*S*)-T4M(1-[β-D-Glc])-OH (20)



A solution of Methyl (*S*)-2-{[(2*S*)-1-(*tert*-butoxy carbonyl) pyrrolidin-2-ylcarbonyl] amino}-3-{1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)[1,2,3]triazol-4-yl}propionate (**70**, Chapter 3) (40 mg, 0.06 mmol) in HCl/EtOAc (2 mL) was stirred for 30 min. Next, the solvent was evaporated *in vacuo* and the crude product was redissolved in MeOH (3 mL), a catalytic amount of K_2CO_3 was added and the mixture was stirred overnight. Purification of the product with an acidic ion exchange column (IRA-120) afforded **20** (16 mg, 0.03 mmol, 53%) as a white solid. Data of rotamers: ^{1}H NMR (400 MHz, D_2O): δ = 7.97 (s, 1H), 5.58 (d, *J* = 9.1 Hz, 1H), 4.28−4.16 (m, 1H), 3.84 (t, *J* = 9.2 Hz, 1H), 3.77 (d, *J* = 11.6 Hz, 1H), 3.67−3.51 (m, 4H), 3.47 (t, *J* = 9.2 Hz, 1H), 3.36−3.09 (m, 4H), 2.34−2.20 (m, 1H), 1.96−1.81 (m, 3H). ^{13}C NMR (75 MHz, D_2O): δ = 172.9, 171.7, 168.9, 142.5, 142.4, 123.2, 86.8, 78.3, 75.4, 71.7, 71.6, 68.4, 59.9, 59.1, 52.7, 52.3, 52.2, 46.0, 45.9, 29.1, 26.5, 25.9, 23.1. HRMS (ESI) *m/z* calculated for $C_{16}H_{26}N_5O_8$ $(M+H)^+$: 416.1781, found: 416.1791.

6.8 REFERENCES

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A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.

-Marie Curie

Reality is merely an illusion, albeit a very persistent one.

-Albert Einstein

Preparation and Evaluation of Glycosylated RGD Derivatives for Integrin Targeting

CHAPTER 7

Abstract*:

A range of cyclic arginine–glycine–aspartate (RGD) derivatives was prepared by a combination of solid phase and solution phase synthesis for selective targeting of $\alpha_v\beta_3$ integrin expressed in tumors. In order to evaluate the value of a triazole moiety as an amide isostere, the side-chain glycosylated cyclic RGD (cRGD) peptides were synthesized with either a natural amide linkage or a triazole. Affinity of the cRGD constructs for $\alpha_v\beta_3$ integrin was determined in a solid phase competitive binding assay, showing strong similarity in binding affinity for each of the compounds under evaluation. Furthermore, the *in vivo* tumor targeting potential of glycosylated cRGD peptides, linked *via* amide or triazole, was investigated by determining the biodistribution of ^{125}I-labeled derivatives in mice with tumors expressing $\alpha_v\beta_3$. All of the cRGD derivatives showed preferential uptake in the subcutaneous tumors, with the highest tumor-to-blood ratio measured for the triazole-linked glycosylated derivative. The results of the present study are a clear indication of the value of the triazole moiety as a suitable amide isostere in the development of glycosylated peptides as pharmaceuticals.

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7.1 INTRODUCTION

With the establishment of mild and effective conditions for the stereoselective synthesis of 1,4-linked triazoles, the potential of triazole to mimic natural amide linkage was put forward. Since then, some precedence for the isosteric value of a triazole has been provided for true peptide analogues,[1,2] but the synthesis of triazole-linked glycopeptides with relevant biological activity has hitherto not been reported. Moreover, to date little is known about the chemical stability of a glycosyl triazole linkage under basic or acidic conditions, with the exception of typical carbohydrate chemistry conditions.[3] In the light of our recently described technology for the preparation of glycopeptides isosteres, it appeared worthwhile to investigate the biological relevance of a triazole function as an amide isostere. Consequently, we became intrigued by the attachment, *via* a triazole, of carbohydrates to the so-called RGD peptides,[4,5] found in proteins of the extracellular matrix such as vitronectin, fibrinogen and laminin.

About 25 years ago the arginine-glycine-aspartic acid (RGD) cell adhesion motif was discovered by Pierschbacher and Ruoslahti[6] in fibronectin. A few years later the vitronectin receptor, or better known as $\alpha_v\beta_3$ integrin,[7] was identified as one of the adhesion molecules recognizing this sequence. The $\alpha_v\beta_3$ integrin, consisting of two subunits (α and β) is expressed on the luminal surface of neovasculature, while it is absent on the quiescent endothelial cells. Integrins link the intracellular cytoskeleton with the extracellular matrix, thereby playing an important role in cell-signaling, cell-cell adhesion, apoptosis and cell-matrix interactions. More specifically, $\alpha_v\beta_3$ integrin is expressed on endothelial cells and modulates cell migration and survival during angiogenesis, while $\alpha_v\beta_3$ integrin expressed on carcinoma cells potentiates metastasis by facilitating extravasation and tissue invasion. In addition, it has been shown that $\alpha_v\beta_3$ integrin is upregulated in tumor blood vessels and is therefore considered as a marker of tumor–induced angiogenesis.[8] Moreover, $\alpha_v\beta_3$ integrin is expressed on various tumor cell types, including ovarian cancer, neuroblastoma, breast cancer and melanoma. As a consequence of the overexpression of $\alpha_v\beta_3$ integrin on cancer cells, the RGD binding site is considered as an attractive pharmaceutical target.[9] As a result, a large number of synthetic RGD analogues have been prepared over the years, which have shown to inhibit the growth of tumor in animal models,[10] presumably by starving the tumors of blood supply. More recently it was reported

that cyclic RGD derivatives, especially the ones consisting of five amino acids (*e.g.* cyclic[Arg-Gly-Asp-D-Phe-Val]), showed a 100-fold better affinity for $\alpha_v\beta_3$ integrin compared to the linear counterparts, with IC_{50} values in the nanomolar range.[11, 12] In particular, Kessler *et al.*[11] showed that until today *cyclo*[Arg-Gly-Asp-D-Phe-Val] is one of the most active and selective antagonists for targeting $\alpha_v\beta_3$ integrin. To study the angionesis and metastasis *in vivo*, Haubner *et al*.[13] synthesized the first radionated peptide, ^{125}I-c(RGDyV). The ^{125}I-c(RGDyV) demonstrated an affinity of 2 nmol/L and high selectivity for $\alpha_v\beta_3$, but was mainly excreted *via* the hepatobiliary route. Conditions to be fulfilled by the ideal radiolabeled $\alpha_v\beta_3$ integrin antagonist include, apart from high affinity and specificity, rapid clearance from the blood (high hydrophilicity) and high metabolic stability. The first two criteria have been extensively studied over the years, while more recently, attempts to produce more hydrophilic cRGD peptides to promote excretion *via* the kidneys have led to glycosylated cRGD peptides.[14] The glycosylated *cyclo*[Arg-Gly-Asp-D-Tyr-Lys(SAA)] showed a longer circulatory half-life in the tumor, greatly reduced liver uptake, and yielded higher tumor-to-blood ratios than the non-glycosylated peptide[4,14] compared with the hydrophobic parent compound. Subsequently, a variety of glycosylated cRGD peptides was prepared by Kessler *et al.* to elucidate the influence of various substitutions on the biological properties.[15] Based on these studies, we hypothesized that triazole isosteres of such glycosylated cRGD peptides would display a comparable pharmaceutical profile.

A strong support for the notion that incorporation of triazoles in natural compounds should not compromise the biological activity was recently provided by van Maarseveen *et al.*[1] by preparation of three triazole containing analogues of the *cyclo*[Pro–Tyr–Pro–Val] (**1**), a naturally occurring tyrosinase inhibitor (Figure 1). As expected, the natural tetrapeptide **1** could not be synthesized due to the problematic ring-closure.[16] However, no problems were encountered in the synthesis of the cyclic triazole analogues **2**-**4**.

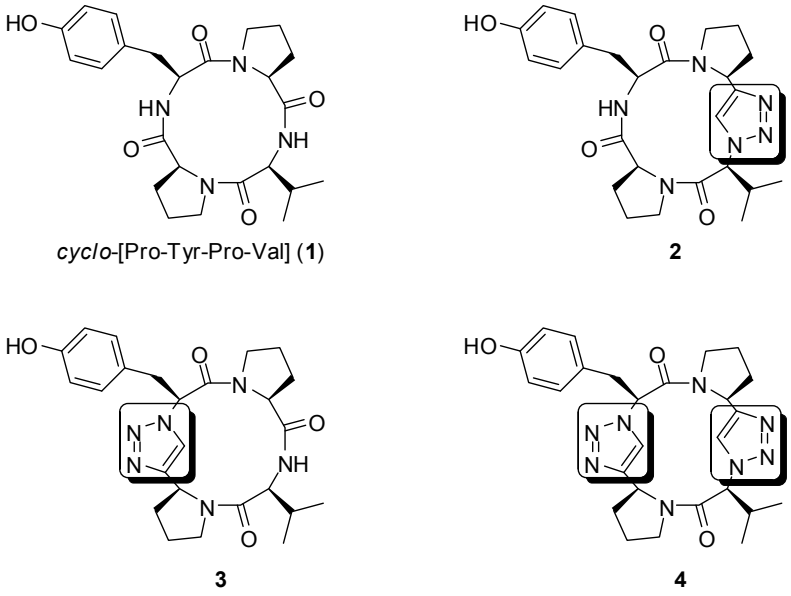


Figure 1. *Cyclo–[Pro–Tyr–Pro–Val] and triazole-containing analogues thereof.*

Next, the inhibitory effect of the cyclotetrapeptide analogues **2-4** on mushroom tyrosinase activity was compared to that of the natural product **1** *via* an *in vitro* spectrophotometric assay, revealing that the triazolyl cyclotetrapeptides not only retained inhibition activity, but in fact, two of the analogues (**2** and **3)** showed an approximately threefold increase in activity as compared to the natural product.

In analogy to the triazolyl tyrosinase inhibitors, studies were initiated by us on triazolyl glycoRGD analogues with two aims. Firstly, to investigate the stability of *N*-triazolyl glycoamino acids and secondly to compare the biological activity with the previously described *N*-glycosylated cRGD peptides. To this end, a representative set of 'regular' RGD peptides as well as two glycosylated RGD derivatives were synthesized by a combination of solid and solution phase techniques. The *in vitro* and *in vivo* $\alpha_v\beta_3$-binding characteristics of these compounds were investigated. In addition, the chemical stability of the triazole linkage in a simple glycosidic amino acid model system was compared with the natural glycosidic amide link found in Nature.

7.2 SYNTHESIS OF GLYCOAMINO ACIDS

Cyclic RGD peptides represent valuable ligands for selective targeting of $\alpha_v\beta_3$ integrins. Consequently, a large number of cyclic RGD analogues has been

prepared[17] and extensively tested. We have shown that 1-azidosugars and acetylene-modified amino acids readily undergo [3+2] cycloaddition *via* a standard protocol for the copper-catalyzed Huisgen addition to form triazole isosteres of glycoamino acids in high yields. Although synthetically of interest, here we explored the potential value of triazolyl glycosylated cRGD peptides as targeting agents. In this respect, the first relevant question to answer is to what extent the triazole analogues of glycoamino acids have increased stability with respect to the natural compounds. Secondly, the medicinal value of the triazole compounds as glycoamino acid mimics can only be evaluated by incorporation in biologically active compounds. For this reason, the amide-linked c[RGDy(*N*-1-β-glyco-Asn)] (**6**) (Figure 2) as well as the triazole-linked glycosylated RGD peptide c(RGDy-T4M(1-[β-D-Glc])) (**5**) were prepared to evaluate the binding affinities for $\alpha_v\beta_3$ integrins. The natural c(RGDfV) (**7**) and c(RGDyV) (**8**) were prepared for reference purposes. D-Tyr was incorporated instead of D-Phe for c[RGDy(*N*-1-β-glyco-Asn)] (**6**), c(RGDy-T4M(1-[β-D-Glc])) (**5**), and c(RGDyV) (**8**) enabling subsequent iodination for tumor imaging as reported by Haubner *et al.*[14]

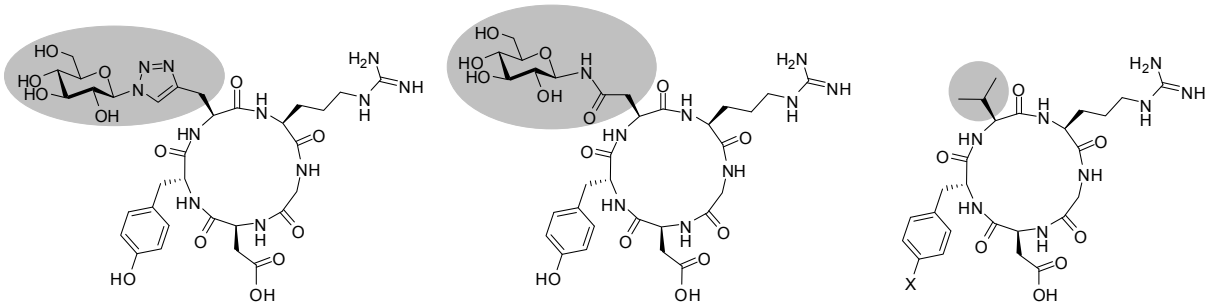
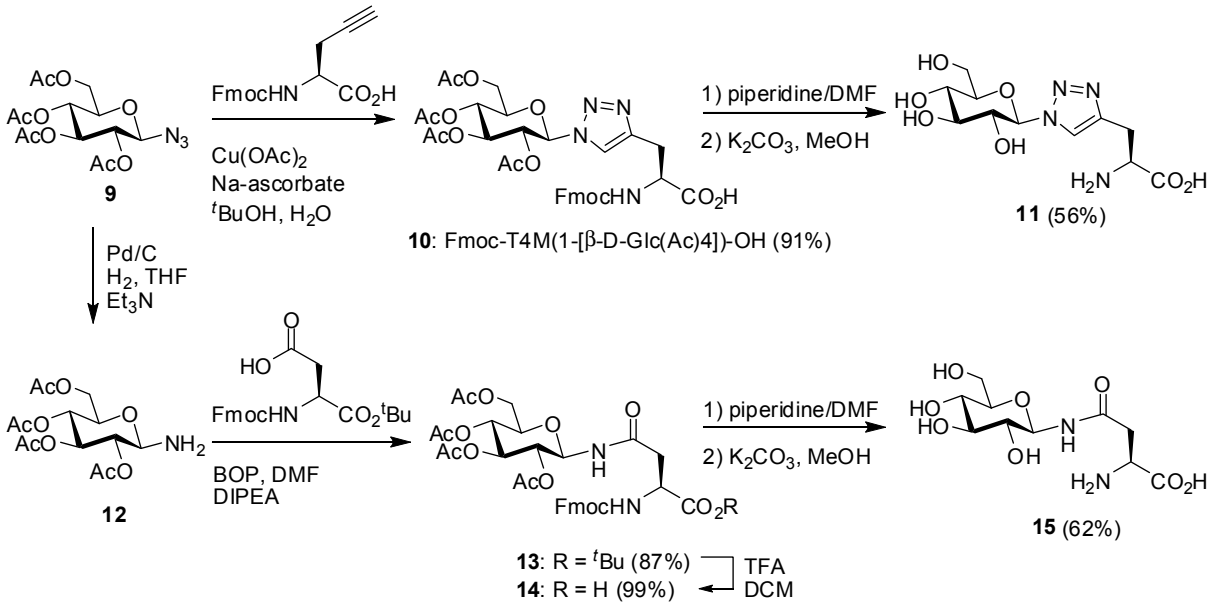


Figure 2. *Cyclic RGD peptides for integrin targeting.*

Since solid phase synthesis of peptides typically involves chain elongation by coupling with Fmoc-protected amino acids, the requisite building blocks for the synthesis of glycoRGD peptides were prepared first. Thus, 1-azido-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose **9**[18] was condensed with Fmoc-protected (*S*)-propargylglycine under the influence of $Cu(OAc)_2$ and sodium ascorbate in a mixture of *tert*-butyl alcohol and water, to give protected Fmoc-T4M(1-[β-D-Glc(Ac)$_4$])-OH (**10**), in 91% yield, Scheme 1. Preparation of the amide-linked glycoamino acid **15** began with hydrogenation of **9**, H_2 and Pd/C in the presence of

Et_3N, to afford β-1-aminoglucose derivative **12**. Subsequent coupling to Fmoc-aspartic acid *tert*-butyl ester yielded acetylated and Fmoc-protected glycosidic asparagine *tert*-butyl ester **13** in a yield of 87%. Next, the *tert*-butyl ester was treated with TFA in DCM (1/1, *v/v*) to afford the free acid Fmoc-[*N*-1-β-glyco(Ac)$_4$-Asn]-OH (**14**) in near quantitative yield.



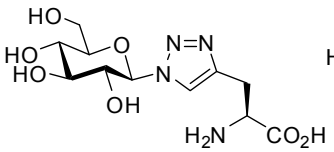
Scheme 1. *Synthesis of glycosylated amino acids **11** and **15**.*

7.3 EVALUATION OF STABILITY

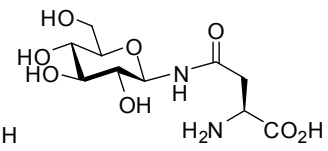
In order to investigate the chemical stability of the substrates T4M(1-[β-D-Glc])-OH (**11**), as well as *N*-1-glycosyl-Asn(OH) (**15**), the protected glycoamino acids **10** and **14** were subjected to treatment with 20% piperidine in DMF (*v/v*) followed by a saponification of the acetyl esters by K_2CO_3 in MeOH. The resulting glycoamino acids T4M(1-[β-D-Glc])-OH (**11**) and *N*-1-glycosyl-Asn(OH) (**15**) were subjected to various acidic and basic conditions as summarized in Table 1.

Table 1. *Stability tests for T4M(1-[β-D-Glc])-OH (**11**) and N-1-β-glyco-Asn (**15**).*

T4M(1-[β-D-Glc])-OH (**11**)



N-1-β-glyco-Asn (**15**)



entry	condities	temperature	time	stability	stability
1	2M HCl (aq)	rt	3 d	++	++
2	2M HCl (aq)	reflux	2 h	++	+
3	4M HCl (aq)	reflux	3 h	++	−−
4	2M NaOH (aq)	rt	3 d	++	−
5	2M NaOH (aq)	reflux	3 h	++	−−
6	2.6M HCl/EtOAc	rt	3 d	++	+
7	Et_3N	rt	4 d	++	++
8	Et_3N	reflux	3 h	++	+
9	$Sn(OTf)_2$, DCM[a]	rt	2 h	++	++

[a]Fmoc- and Ac-protected derivatives were used.

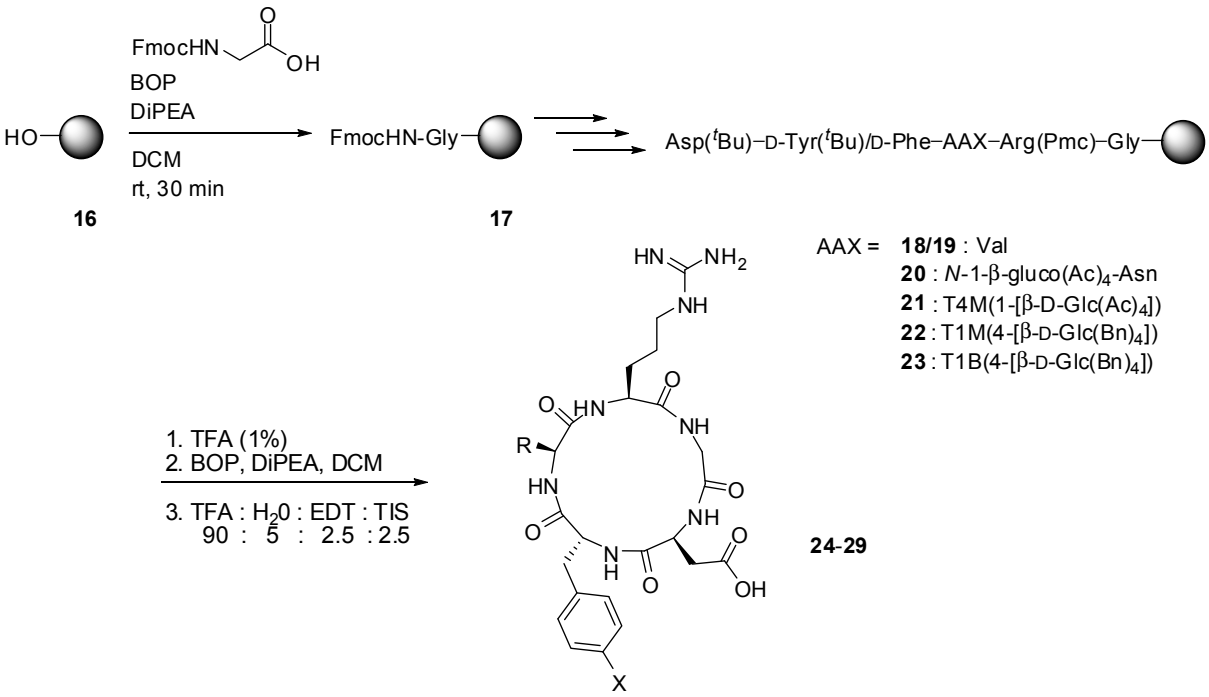
First of all, it became apparent that the natural and the triazole-linked glycoamino acids were both remarkably resistant to 2 M aqueous hydrochloric acid. However, at elevated temperature, the hydrolysis of the amide-linked glycoamino acid **15** began to take place, while the triazole-linked T4M(1-[β-D-Glc])-OH **11** appeared completely robust. Similarly, triazole **11** was also found to be fully compatible with basic conditions, in strong contrast with the rapid hydrolysis of the natural amide-linked *N*-1-β-glyco-Asn (**15**). From these results, it can be concluded that triazole-linked substrates are versatile building blocks for the preparation of stable analogues of the common asparagine-linked *N*-glycoproteins.

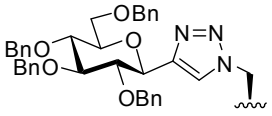
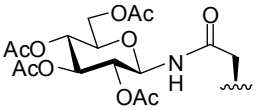
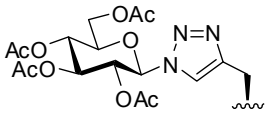
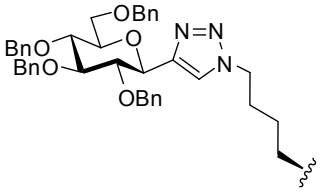
7.4 PREPARATION OF GLYCOSYLATED CYCLIC RGD PEPTIDES VIA SOLID PHASE SYNTHESIS

For the cyclic RGD peptides, a solid phase strategy was chosen for synthesis of the peptide chain, followed by cleavage from the resin prior to cyclization. In order to avoid racemization of the carbonyl α-carbon during cyclization, Fmoc-glycine was

elected as *C*-terminal amino acid and coupled to trityl resin with DiPEA in DCM as depicted in Scheme 2. Subsequent elongation of the peptide was executed by application of standard Fmoc chemistry, involving HOBt/DIPCDI-mediated coupling followed by piperidine-induced Fmoc deprotection.

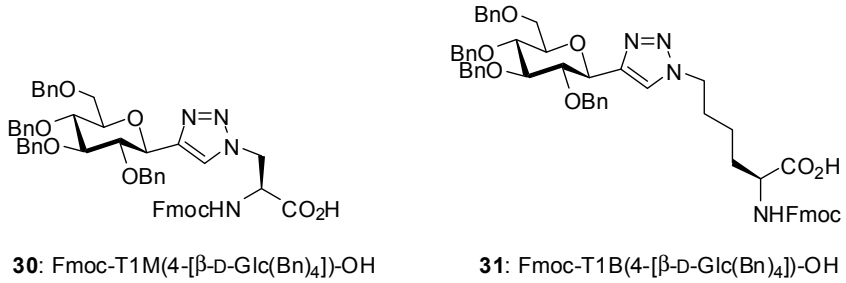
Scheme 2. *Preparation of the RGD peptides.*



RGD	X	R	RGD	X	R
24	H				
25	OH		**28**	OH	
26	OH				
27	OH		**29**	OH	

Besides the amino acids **10** and **14** two different *C*-triazole-linked amino acids (**30**[19] and **31**[19], Figure 3) were incorporated. All four amino acids, Fmoc-T4M(1-[β-D-

Glc(Ac)$_4$])-OH (**10**), T1M(4-[β-D-Glc(Bn)4])-OH (**30**), Fmoc-T1B(4-[β-D-Glc(Bn)4])-OH (**31**) and Fmoc-[*N*-1-β-glyco(Ac)$_4$]-Asn(OH) (**14**) were compatible with the coupling conditions, although the amide-linked glycoamino acid was incorporated at a much slower rate.



30: Fmoc-T1M(4-[β-D-Glc(Bn)$_4$])-OH **31**: Fmoc-T1B(4-[β-D-Glc(Bn)$_4$])-OH

Figure 3. *T1M(4-[β-D-Glc(Bn)4])-OH (**30**) and T1B(4-[β-D-Glc(Bn)4])-OH (**31**).*

After completion of the synthesis, the fully protected pentapeptides were cleaved from the resin using a 1% solution of TFA in DCM and purified by extraction and/or silica gel column chromatography. Next, cyclization of the linear RGD peptides was effected under the influence of BOP to give the protected cyclic peptides **24**-**29**. Since the latter glycopeptides still carry protective groups on the carbohydrate moiety, attempts were undertaken to deprotect the benzylated RGD peptides **28** and **29**. Surprisingly, a range of debenzylation conditions failed to produce the desired product in acceptable yield (Table 2).

Table 2. *Attempted reaction conditions for the hydrogenation of the benzyl protection groups.*

entry	conditions	product
1	Pd/C, MeOH, H_2 (1bar)	0%
2	Pd/C, MeOH, H_2 (3bar)	0%[a]
3	Pd/C, "Degussa", MeOH H_2 (1bar)	0%
4	$Pd(OH)_2$, MeOH, H_2 (1bar)	0%
5	$Pd(OH)_2$ "Degussa", MeOH, H_2 (1bar)	0%
6	Ra-Ni, H_2O, H_2 (1bar)	0%[a]
7	BF_3·OEt, EtOH	<10%

[a]Only partially deprotected products were formed.

Fortunately, the acetyl-protected carbohydrates **26** and **27** were easily deprotected *via* treatment with a K_2CO_3 solution, affording the desired glycosylated cRGD peptides **5** and **6** in good overall yield (14-18%). Finally, the glycosylated cRGD peptides depicted in Figure 2 (**5** and **6**) as well as c(RGDyV) (**8**) and c(RGDfV) (7) were purified by semi-preparative RP-HPLC and examined *in vitro* and *in vivo* to study the $\alpha_v\beta_3$ receptor affinities.

7.5 SOLID PHASE $\alpha_v\beta_3$ BINDING ASSAY

The affinities of c(RGDfV) (**7**) , c(RGDyV) (**8**), c(RGDy-[*N*-1-β-gluco-Asn]) (**6**), and c(RGDy-T4M(1-[β-D-Glc])) (**5**) for the $\alpha_v\beta_3$ integrin as determined in the competitive binding assay are summarized in Figure 4.

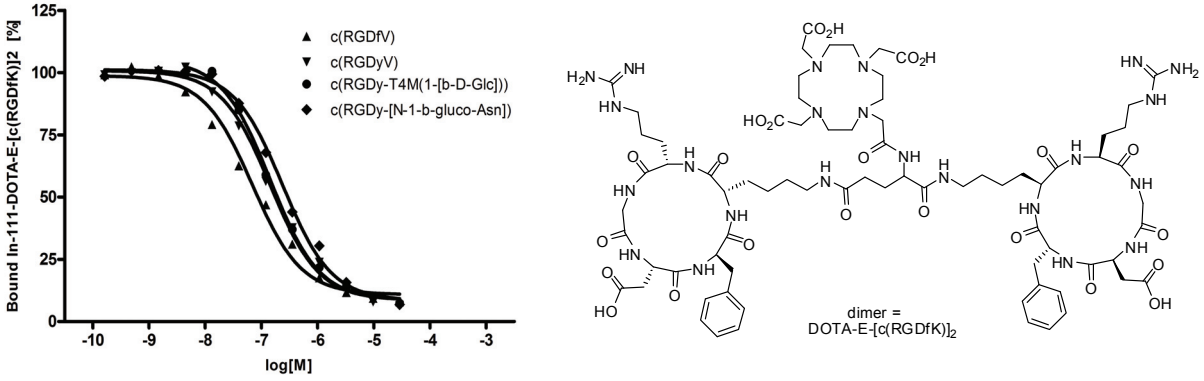


Figure 4. *Competition of specific binding of ^{111}In-DOTA-E-[c(RGDfK)]$_2$ with c(RGDfV) (▲), c(RGDyV) (▼), c(RGDy-T4M(1-[β-D-Glc])) (●) and c(RGDy-[N-1-β-gluco-Asn]) (◆).*

From Figure 4, it becomes clear that binding of ^{111}In-DOTA-E-[c(RGDfK)]$_2$[20] to $\alpha_v\beta_3$ was inhibited by each compound in a concentration dependent manner. In fact, only relatively small differences between the different peptides could be observed, with IC_{50} values of 65 nM for c(RGDfV), 144 nM for c(RGDyV), 238 nM for c(RGDy-[*N*-1-β-gluco-Asn]), and 144 nM for c(RGDy-T4M(1-[β-D-Glc])), indicating that side-chain modification had only limited effect on the $\alpha_v\beta_3$ binding affinity of the compounds, which is in agreement with earlier reports.[15, 21] In addition, these data showed that only a slight difference was noticeable between the carbohydrate containing RGD peptides (c(RGDy-T4M(1-[β-D-Glc])) and c(RGDy-[*N*-1β-gluco-Asn]), which can be a

first indication that the glycoamino acid binding characteristics are nearly unchanged upon substitution of the amide linkage for a triazole linkage.

7.6 BIODISTRIBUTION STUDIES

Side-chain glycosylation can improve the pharmacological properties of hydrophobic/lipophilic peptides,[22] which has also been demonstrated for RGD.[14] In order to validate the potential of the glycoamino acids **11** and **15** for incorporation in glycoRGD peptides, the biodistribution of ^{125}I-c(RGDyV), ^{125}I-c(RGDy-[*N*-1-β-gluco-Asn]), and ^{125}I-c(RGDy-T4M(1-[β-D-Glc])) in athymic mice with subcutaneous (s.c.) $\alpha_v\beta_3$-expressing tumors was determined. The results of the biodistribution studies of ^{125}I-RGDyV, ^{125}I-c(RGDy-[*N*-1-β-gluco-Asn]), and ^{125}I-c(RGDy-T4M(1-[β-D-Glc])) in athymic mice with SK-RC-52 tumors at 2 h postinjection (p.i.) are summarized in Table 3 and Figure 5. In order to determine the non-specific uptake of the peptides, the biodistribution was also determined in the presence of excess unlabeled RGD compound, *i.e.* dimeric DOTA-E-[c(RGDfK)]$_2$.

Table 3. *Biodistribution data of ^{125}I-c(RGDyV), ^{125}I- c(RGDy-[N-1-β-gluco-Asn]), and ^{125}I- c(RGDy-T4M(1-[β-D-Glc])) in the presence and absence of an excess of unlabeled DOTA-E-[c(RGDfK)]$_2$ in athymic mice with s.c. SK-RC-52 tumors 2 h after injection. The organ uptake is expressed as %ID/g.*

	^{125}I-c(RGDyV)		^{125}I-c[RGDy-(*N*-1-β-gluco-Asn)]		^{125}I-c[RGDy-T4M(1-[β-D-Glc]))	
		non-specific uptake		non-specific uptake		non-specific uptake
Blood	0.13 ± 0.01	0.26 ± 0.12	0.30 ± 0.04	0.28 ± 0.06	0.09 ± 0.04	0.10 ± 0.10
Muscle	0.17 ± 0.08	0.08 ± 0.03	0.45 ± 0.18	0.28 ± 0.24	0.17 ± 0.04	0.06 ± 0.04
Tumor	0.77 ± 0.10	0.34 ± 0.07	2.63 ± 0.45	0.89 ± 0.17	1.31 ± 0.33	0.51 ± 0.01
Lung	0.36 ± 0.08	0.29 ± 0.09	0.74 ± 0.10	0.40 ± 0.07	0.29 ± 0.03	0.13 ± 0.02
Spleen	0.19 ± 0.02	0.20 ± 0.13	1.07 ± 0.07	0.43 ± 0.05	0.46 ± 0.06	0.18 ± 0.04
Kidney	0.84 ± 0.04	0.71 ± 0.24	1.89 ± 0.07	1.43 ± 0.27	1.27 ± 0.32	0.99 ± 0.20
Liver	1.07 ± 0.11	1.49 ± 0.44	0.76 ± 0.05	0.36 ± 0.05	0.37 ± 0.07	0.22 ± 0.05
Intestine	1.93 ± 1.62	1.82 ± 0.36	2.10 ± 0.44	1.15 ± 0.78	0.80 ± 0.20	0.30 ± 0.13

All peptides ^{125}I-c(RGDyV), ^{125}I-c(RGDy-[*N*-1-β-gluco-Asn]), and ^{125}I-c(RGDy-T4M(1-[β-D-Glc])) rapidly cleared from the blood as blood levels 2 h p.i. did not exceed 0.3% ID/g. Two hours after injection, the concentration of ^{125}I-c(RGDy-[*N*-1-β-gluco-Asn])

and ^{125}I-c(RGDy-T4M(1-[β-D-Glc])) in the tumor was the highest of all tissues examined. Co-injection of excess unlabeled DOTA-E-[c(RGDfK)]$_2$ (50 μg) along with ^{125}I-c(RGDyV), ^{125}I-c(RGDy-[*N*-1-β-gluco-Asn]), and ^{125}I-c(RGDy-T4M(1-[β-D-Glc])) indicated that for each of the three compounds, the major part of the uptake in the tumor was $\alpha_v\beta_3$-mediated.

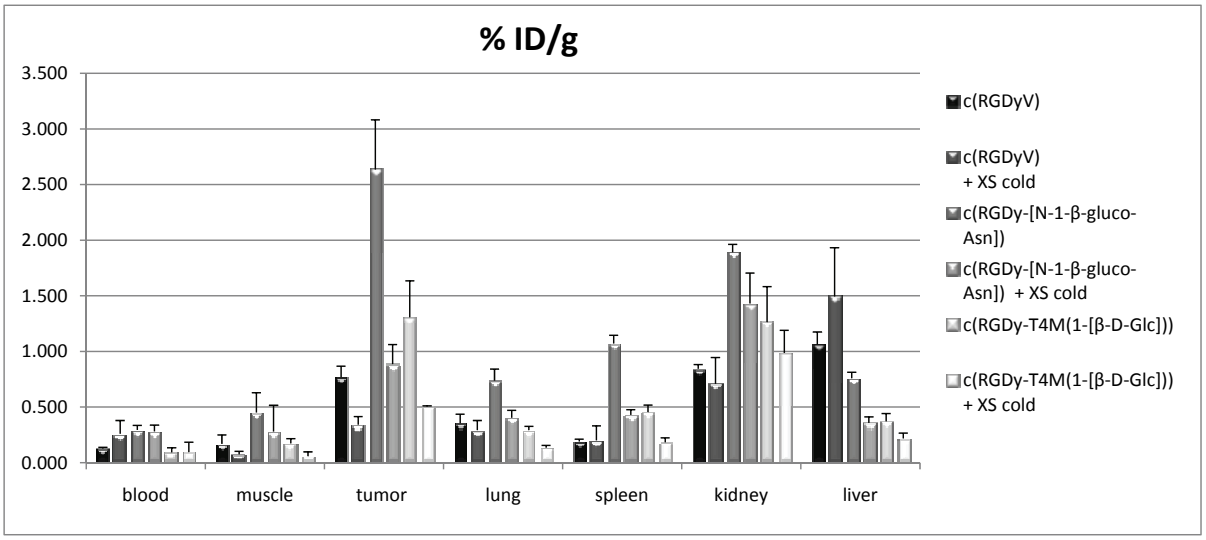


Figure 5. *Biodistribution of RGD peptides in athymic mice.*

In this mouse model ^{125}I-c(RGDy-T4M(1-[β-D-Glc])) revealed the highest tumor-to-blood ratio (18 at 2 h p.i.). The carbohydrate bearing cRGD peptides, ^{125}I-c(RGDy-[*N*-1-β-gluco-Asn]), and ^{125}I-c(RGDy-T4M(1-[β-D-Glc])) also showed $\alpha_v\beta_3$-mediated uptake in non-target organs like lung, spleen, and intestine. Specific uptake of cRGD analogues in normal tissues has been reported in previous studies and is probably due to $\alpha_v\beta_3$ expression in these tissues.[23] Furthermore, the conducted studies once more show that tumor uptake is not solely dependent on the binding affinity but relies on many more factors such as blood residence time, molecular weight, structure, charge etc. These aspects may explain the fact that ^{125}I-c(RGDy-[*N*-1-β-gluco-Asn]) displayed the highest tumor uptake while the corresponding monomer failed to provide the highest binding affinity. Although ^{125}I-c(RGDy-T4M(1-[β-D-Glc])) showed the highest tumor-to-blood ratio, it showed a lower tumor uptake than ^{125}I-c(RGDy-[*N*-1-β-gluco-Asn]). Therefore, it may be concluded that the high tumor uptake of ^{125}I-c(RGDy-[*N*-1-β-gluco-Asn]) can only partially be explained by the presence of the carbohydrate moiety.

7.7 CONCLUSION

A high yielding route for the preparation of Fmoc-T4M(1-[β-D-Glc(Ac)$_4$])-OH starting from azidoglucose and Fmoc-protected propargylglycine is described by application of our technology for the synthesis of triazolyl glycoamino acids. The T4M building block displayed significantly improved chemical stability with respect to the 'natural' amide-linked glycoamino acid, and is fully compatible with basic and acidic conditions. The incorporation of the Fmoc-protected T4M derivative in an RGD peptide proceeded without incidence, in fact more smoothly than the amide-linked glycoamino acids, with the final yield of the substrate being comparable to that of the other RGD substrates. The solid phase binding assay revealed no significant differences between the different peptides under evaluation confirming that modification of the side-chain of the RGD peptide did not affect affinity for $\alpha_v\beta_3$. The ^{125}I-labeled cyclic peptides accumulated specifically in SK-RC52 tumors. The uptake of the cyclic RGD substrates in the tumor, lung, spleen, and intestine were $\alpha_v\beta_3$-mediated. The biodistribution studies indicated that the side-chain glycosylated RGD peptide ^{125}I-c(RGDy-T4M(1-[β-D-Glc])) showed improved pharmacological properties as compared to ^{125}I-c(RGDyV) with reduced liver uptake and highest uptake in the tumor. Moreover, the *in vitro* and *in vivo* tests indicated that c(RGDy-T4M(1-[β-D-Glc])) to some extent has the same pharmacological properties as c(RGDy-[*N*-1-β-gluco-Asn]), which may be interpreted as one of the first examples of the value of triazoles as amide isosteres. The ease of synthesis of the triazolyl glycoamino acids, the improved stability and behavior in solid phase peptide synthesis and the similar pharmacological properties comprise a demonstration of the potential of T4M building blocks as isosteres of amide-linked glycoamino acids for the synthesis of pharmaceutically relevant glycopeptide mimics.

7.8 ACKNOWLEDGMENT

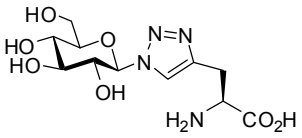
Margot J. A. Seegers is kindly acknowledged for her contribution to this chapter. Prof. dr. Otto C. Boerman, Annemieke C. Soede and Dr. Peter Laverman (Department of Nuclear Medicine, Radboud University Nijmegen Medical Center, The Netherlands) are gratefully acknowledged for conducting the solid phase $\alpha_v\beta_3$ binding assay and biodistribution studies and fruitful discussions. Stan Groothuys is acknowledged for the preparation of compounds **30** and **31**.

7.9 EXPERIMENTAL

General information

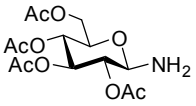
Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Merck Hitachi HPLC using an analytical reversed-phase column (Alltech Adsorbosphere C18, 300 Å, 5 μm, 4.6 mm x 250 mm) and a Merck Hitachi (L-4000) UV detector operating at 215 nm. Elution was effected using an appropriate gradient from 0.1% trifluoroacetic acid (TFA) in H_2O/MeCN (95/5, v/v) to 0.1% TFA in MeCN/H_2O (95/5, v/v). Semi-preparative HPLC was performed using an Alltech Adsorbosphere XL C18 column (250 x 10 mm, pore size 100 Å, particle size 10 μm) at a flow rate of 5.0 mL/min. For other general information see Section 2.6.

(*S*)-T4M(1-[β-D-Glc])-OH (11).



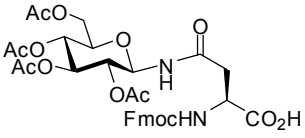
Fmoc-(*S*)-T4M(1-[β-D-Glc(Ac)$_4$])-OH **(10)** (500 mg, 0.70 mmol) was treated with 20% piperidine in DMF (*v/v*, 5 mL) and stirred for 20 min before concentration of the solvent *in vacuo*. The crude product was dissolved in MeOH (5 mL), a catalytic amount of K_2CO_3 was added and the mixture was stirred for 1 h. Purification of the product with a basic ion exchange column (IRA-410) afforded the triazolyl-linked glycosidic amino acid **3** (125 mg, 0.39 mmol, 56%) as a white solid. IR (film): ν 3309, 2362, 2327, 1623 cm^{-1}. ^{1}H NMR (400 MHz, D_2O): δ = 5.01 (d, *J* = 8.4 Hz, 1H), 4.04 (s, 1H), 3.89 (d, *J* = 12.2 Hz, 1H), 3.74 (dd, *J* = 13.0, 4.8 Hz, 1H), 3.46−3.39 (m, 2H), 3.44 (m, 2H), 3.14−2.80 (m, 2H). ^{13}C NMR (75 M Hz, $CDCl_3$): δ =172.3, 141.7, 123.3, 86.9, 78.4, 75.4, 71.7, 68.4, 59.9, 53.7, 25.7. HRMS (ESI) *m/z* calculated for $C_{34}H_{37}N_4O_{13}$ $(M+H)^+$: 709.2357, found: 709.2344.

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl amine (12).



The glucopyranosyl amine was prepared according to a literature procedure[24] with slight modification. In an oven-dried Schlenk flask filled with N_2, the azidoglucose **9** (500 mg, 1.34 mmol), Et_3N (140 mg, 1.4 mmol) and Pd/C (14 mg, 0.1 mmol) were suspended in THF (3 mL). Next the Schlenk flask was subjected to a sequence of vacuum and flushing with H_2 (3 ×) after which it was vigorously stirred while bubbling H_2 through the solution. After 30 min, the reaction was completed, the slurry was filtrated over Hyflo and THF was evaporated under reduced pressure to yield **4** as a white solid (455 mg, 1.31 mmol, 98%), which was sufficiently pure for further reactions. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.22 (t, *J* = 9.3 Hz, 1H), 5.02 (t, *J* = 9.3 Hz, 1H), 4.83 (d, *J* = 9.2 Hz, 1H), 4.21 (d, *J* = 9.2 Hz, 1H), 4.10 (dd, *J* = 12.4, 2.1 Hz, 1H), 3.65 (ddd, *J* = 9.8, 4.6, 2.1 Hz, 1H), 2.10 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H). Spectral data are in accordance with literature.[18]

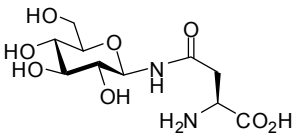
Fmoc-(*S*)-(*N*-1-Glu(Ac)$_4$-Asn)-OH (14).



The amide-linked sugar amino acid was prepared from glucopyranosyl amine **12** and Fmoc-Asp-O^{t}Bu *via* a procedure reported by van Ameijde *et al.*[25] involving peptide coupling catalyzed by BOP followed by deprotection using TFA/DCM (4 h, rt) to afford the *N*-glycosylasparagine **14** as a white solid (798 mg, 1.17 mmol, 86%). R_f = 0.12 (EtOAc/heptane 2:1). IR (film): ν 3313, 2250, 1740, 1506 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ =

7.76 (d, *J* = 7.5 Hz, 2H), 7.58 (d, *J* = 7.4 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 6.70 (d, *J* = 8.8 Hz, 1H), 6.16 (d, *J* = 7.6 Hz, 1H), 5.33 (t, *J* = 9.5 Hz, 1H), 5.26 (t, *J* = 9.1 Hz, 1H), 5.08 (t, *J* = 9.7 Hz, 2H), 4.94 (t, *J* = 9.5 Hz, 1H), 4.67−4.53 (m, 1H), 4.47−4.34 (m, 2H), 4.30 (dd, *J* = 12.6, 4.4 Hz, 1H), 4.22 (t, *J* = 7.04 Hz, 1H), 4.08 (dd, *J* = 12.6, 2.0 Hz, 1H), 3.82 (ddd, *J* = 10.1, 4.1, 2.0 Hz, 1H), 2.90 (dd, *J* = 16.3, 2.8 Hz, 1H), 2.76 (dd, *J* = 16.3, 5.2 Hz, 1H), 2.06 (s, 5H), 2.04 (s, 3H), 2.02 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 176.1, 173.9, 171.4, 170.9, 170.2, 169.8, 156.6, 143.7, 141.3, 127.9, 127.2, 125.3, 120.1, 78.0, 73.8, 272.9, 70.8, 68.2, 67.6, 61.8, 50.5, 47.1, 37.7, 20.8, 20.7, 20.7, 20.6. HRMS (ESI) *m/z* calculated for $C_{33}H_{36}N_2NaO_{14}$ $(M+Na)^+$: 707.2051, found: 707.2064.

(*S*)-*N*-1-Gluco-Asn (15).



Fmoc-(*N*-1-Glu(Ac)$_4$-Asn)-OH (**14**, 500 mg, 0.73 mmol) was treated with 20% piperidine in DMF (*v/v*, 5 mL) and stirred for 20 min, before concentration of the solvent *in vacuo*. The crude product was dissolved in MeOH (5 mL), a catalytic amount of K_2CO_3 was added and the mixture was stirred for 1 h. Purification of the product with a basic ion exchange column (IRA-410) afforded the amide-linked glycosidic aspargine **7** (133 mg, 0.45 mmol, 62%) as a white solid. ^{1}H NMR (400 MHz, D_2O): δ = 8.14 (s, 1H), 5.77 (d, *J* = 9.2 Hz, 1H), 4.10 (t, *J* = 5.0 Hz, 1H), 4.02 (t, *J* = 9.2 Hz, 1H), 3.93 (d, *J* = 11.4 Hz, 1H), 3.82−3.71 (m, 3H), 3.64 (t, *J* = 9.1 Hz, 1H), 3.43−3.34 (m, 1H). HRMS (ESI) *m/z* calculated for $C_{33}H_{36}N_2O_{14}$ $(M+H)^+$: 707.2064, found: 707.2051.

General procedure A for the synthesis of the RGD peptides.
Loading of the trityl resin, synthesis of the pentapeptides, cleavage, subsequent cyclization and deprotection of the cyclic RGD peptides were carried out following standard Fmoc peptide chemistry protocols. Side-chain protection was 2,2,5,7,8-pentamethylchromane (Pmc) for arginine and *tert*-butyl (^{t}Bu) for aspartic acid and tyrosine.

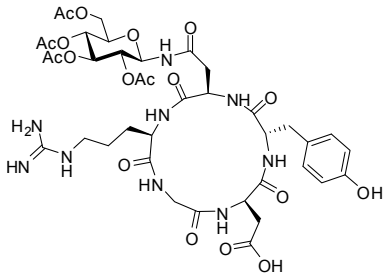
General procedure B for side chain protecting group removal and resin cleavage.
The peptides were treated with a solution of 95% trifluoracetic acid (TFA), 2.5% water, and 2.5% ethanedithiol (EDT) for 4 h at ambient temperature. Next, the glycopeptides were precipitated in diethyl ether. The crude product was isolated by centrifugation and washed three times with diethyl ether. Purification by extraction and/or silica gel column chromatography afforded white solids.

General procedure C for acetyl removal on the carbohydrates.
The crude product was dissolved in MeOH, a catalytic amount of K_2CO_3 was added and the mixture was stirred for 1 h. Ion exchange resin (Amberlyte IR-120) was added until the solution was neutral and the ion exchange resin was filtered off. Evaporation of the solvent *in vacuo* provided the products as white solids. A final purification for the biological studies was performed *via* semi-preparative RP-HPLC as described above.

c(RGDfV) (7)
Preparation according to general procedures A and B afforded **7** (105 mg, 0.18 mmol, 16% relative to the resin). Rt (min): 8.1. HRMS (ESI) *m/z* calculated for $C_{26}H_{39}N_8O_7$ $(M+H)^+$: 575.2914, found: 575.2942.

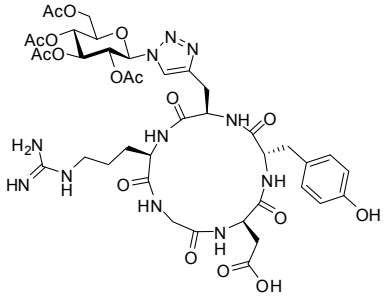
c(RGDyV) (8)
Preparation according to general procedures A and B afforded **7** (85 mg, 0.14 mmol, 13% relative to the resin). Rt (min): 8.2. HRMS (ESI) *m/z* calculated for $C_{26}H_{39}N_8O_8$ $(M+H)^+$: 591.2891, found: 591.2891.

c(RGDy-*N*-[1-β-gluco(OAc)$_4$]-Asn) (26)



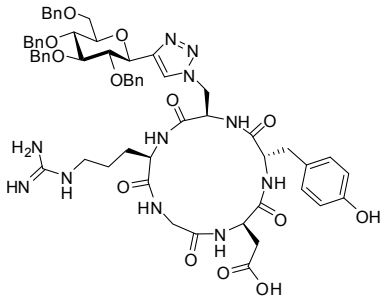
Preparation according to general procedures A and B afforded **26** (60 mg, 0.06 mmol, 17% relative to the resin). ^{1}H NMR (400 MHz, MeOD): δ = 7.02 (d, *J* = 8.6 Hz, 2H), 6.70 (d, *J* = 8.6 Hz, 2H), 5.32 (t, *J* = 9.5 Hz, 1H), 5.23 (t, *J* = 9.4 Hz, 1H), 5.04 (t, *J* = 10.0 Hz, 1H), 4.95 (t, *J* = 9.5 Hz, 1H), 4.73 (t, *J* = 6.9 Hz, 1H), 4.57−4.52 (m, 1H), 4.44−4.39 (m, 1H), 4.28−4.23 (m, 3H), 4.11 (dd, *J* = 12.4 Hz, 2.2 Hz, 1H), 3.92 (ddd, *J* = 10.2 Hz, 4.6 Hz, 2.4 Hz, 1H), 3.25−3.13 (m, 2H), 3.00−2.95 (m, 1H), 2.86−2.76 (m, 2H), 2.68 (dd, *J* = 16.1 Hz, 6.9 Hz, 1H), 2.57 (dd, *J* = 16.5 Hz, 6.8 Hz, 1H), 2.48 (dd, *J* = 16.1 Hz, 5.3 Hz, 1H), 2.03 (s, 3H), 2.03 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.98−1.55 (m, 4H). LRMS *m/z* calculated for $C_{39}H_{54}N_9O_{18}$ $(M+H)^+$: 936.4, found: 936.4.

c(RGDy-T4M(1-[β-D-Glc(Ac)$_4$])) (27)



Preparation according to general procedures A and B afforded **27** (48 mg, 0.05 mmol, 14% relative to the resin). ^{1}H NMR (400 MHz, MeOD): δ = 7.94 (s, 1H), 6.99 (d, *J* = 8.5 Hz, 2H), 6.69 (d, *J* = 8.5 Hz, 2H), 6.09 (d, *J* = 9.2 Hz, 1H), 5.63 (t, *J* = 9.4 Hz, 1H), 5.53 (t, *J* = 9.4 Hz, 1H), 5.27 (t, *J* = 9.8 Hz, 1H), 4.71 (dd, *J* = 8.3 Hz, 5.7 Hz, 1H), 4.58−4.50 (m, 2H), 4.35−4.24 (m, 3H), 4.17 (dd, *J* = 12.3 Hz, 1.7 Hz, 1 H), 4.13−4.09 (m, 1H), 3.38 (d, *J* = 15.4 Hz, 1H), 3.26−3.18 (m, 3H), 3.12−3.05 (m, 1H), 2.99−2.94 (m, 1H), 2.84−2.77 (m, 2H), 2.56 (dd, *J* = 16.6 Hz, 5.8 Hz, 1H), 2.06 (s, 3H), 2.00 (s, 3H), 2.00 (s, 3H), 1.83 (s, 3H), 2.00−1.52 (m, 4H). LRMS *m/z* calculated for $C_{40}H_{54}N_{11}O_{17}$ $(M+H)^+$: 960.4, found: 960.4.

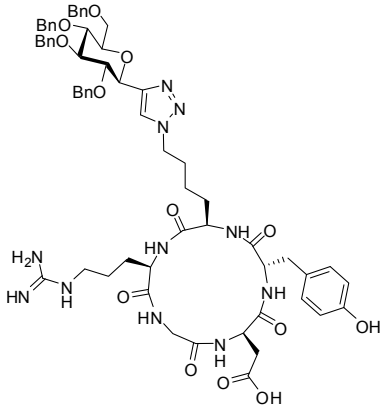
c(RGDy-T1M(4-[β-D-Glc(Bn)$_4$])) (28)



Preparation according to general procedures A and B afforded **28**. ^{1}H NMR (400 MHz, MeOD): δ = 7.44 (d, *J* = 2.1 Hz, 1H), 7.34−6.98 (m, 20H), 6.92 (d, *J* = 8.6 Hz, 2H), 6.72 (d, *J* = 8.6 Hz, 2H), 4.90 (ap s, 1H), 4.71 (t, *J* = 7.1 Hz, 1H), 4.66−4.42 (m, 7H), 4.34−4.20 (m, 3H), 4.16 (dd, *J* = 8.7, 5.4 Hz, 1H), 3.93−3.65 (m, 7H), 3.25−3.06 (m, 3H), 2.98−2.68 (m, 4H), 2.56 (dd, *J* = 16.5, 6.7 Hz, 1H), 1.91−1.43 (m, 5H). HRMS

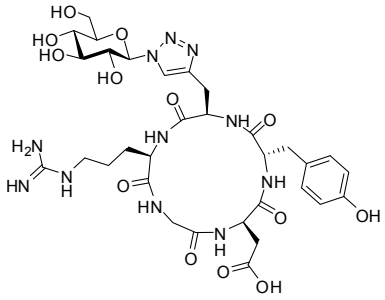
(ESI) *m/z* calculated for $C_{60}H_{70}N_{11}O_{13}$ $(M+H)^+$: 1152.5155, found: 1152.5122. HRMS (ESI) *m/z* calculated for $C_{60}H_{69}N_{11}O_{13}Na$ $(M+Na)^+$: 1174.4974, found: 1174.5026.

c(RGDy-T1B(4-[β-D-Glc(Bn)$_4$])) (29)



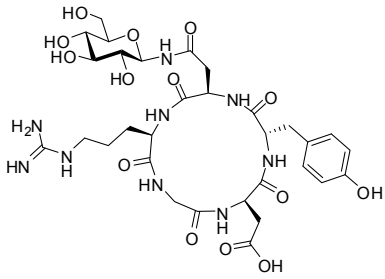
Preparation according to general procedures A and B afforded **29**. ^{1}H NMR (400 MHz, DMSO-D6): δ = 7.44 (ap s, 1H), 7.31−6.99 (m, 20H), 6.78−6.57 (m, 4H), 6.56 (d, *J* = 8.4 Hz, 1H), 4.84−4.61 (m, 5H), 4.53−4.24 (m, 7H), 4.15−3.98 (m, 2H), 3.77−3.69 (m, 4H), 3.60−3.51 (m, 2H), 2.88−2.66 (m, 4H), 2.40−2.29 (m, 3H), 1.79−1.35 (m, 6H). HRMS (ESI) *m/z* calculated for $C_{63}H_{76}N_{11}O_{13}$ $(M+H)^+$: 1194.5624, found: 1194.5660. HRMS (ESI) *m/z* calculated for $C_{63}H_{75}N_{11}O_{13}Na$ $(M+Na)^+$: 1216.5443, found: 1216.5521.

c(RGDy-T4M(1-[β-D-Glc])) (5)



Preparation from c(RGDy-T4M(1-[β-D-Glc(Ac)$_4$])) (**27**) according to general procedure C for the removal of the acetyl groups afforded **5** (39 mg, 0.05 mmol, 99%). Rt (min): 7.9. ^{1}H NMR (400 MHz, D_2O): δ = 7.83 (s, 1H), 7.01 (d, *J* = 8.4 Hz, 2H), 6.68 (d, *J* = 8.4 Hz, 2H), 5.72 (d, *J* = 9.2 Hz, 1H), 4.68 (t, *J* = 7.1 Hz, 1H), 4.51 (dd, *J* = 9.9, 5.9 Hz, 1H), 4.43 (t, *J* = 6.9 Hz, 1H), 4.34 (dd, *J* = 8.9 Hz, 5.6 Hz, 1H), 4.21 (d, *J* = 14.9 Hz, 1H), 3.99 (t, *J* = 9.2 Hz, 1H), 3.94 (d, *J* = 10.4 Hz, 1H), 3.68−3.55 (m, 3H), 3.64 (t, *J* = 9.3 Hz, 1H), 3.50 (d, *J* = 14.9 Hz, 1H), 3.09−2.95 (m, 3H), 3.04 (dd, *J* = 15.3, 6.5 Hz, 1H), 2.94 (dd, *J* = 13.4, 5.9 Hz, 1H), 2.86 (dd, *J* = 13.2, 10.2 Hz, 1H), 2.67 (dd, *J* = 15.8, 7.0 Hz, 1H), 2.53 (dd, *J* = 15.7, 7.3 Hz, 1H), 1.75−1.63 (m, 1H), 1.55−1.42 (m, 1H), 1.35−1.23 (m, 2H). HRMS (ESI) *m/z* calculated for $C_{32}H_{46}N_{11}O_{13}$ $(M+H)^+$: 792.3210, found: 792.3277.

c(RGDy-*N*-[1-gluco]-Asn) (6)



Preparation from c(RGDy-*N*-[1-β-gluco(OAc)$_4$]-Asn) (**26**) according to general procedure C for the removal of the acetyl groups according to procedure C afforded **6** (50 mg, 0.06 mmol, 99%). Rt (min): 6.3. ^{1}H NMR (400 MHz, D_2O/MeOD): δ = 7.01 (d, *J* = 8.4 Hz, 2H), 6.68 (d, *J* = 8.2 Hz, 2H), 4.88 (ap. d, *J* = 9.5 Hz, 1H), 4.70 (t, *J* = 7.2 Hz, 1H), 4.50 (dd, *J* = 6.8 Hz, 5.0 Hz, 1H), 4.45−4.38 (m, 2H), 4.24 (d, *J* = 14.5 Hz, 1H), 3.89 (ap. d, *J* = 11.1 Hz, 1H), 3.74 (dd, *J* = 12.4 Hz, 5.1 Hz, 1H), 3.58 (t, *J* = 9.2 Hz, 1H), 3.53−3.40 (m, 3H), 3.38−3.31 (m, 1H), 3.26−3.15 (m, 2H), 2.98 (dd, *J* = 13.3 Hz, 5.9 Hz, 1H), 2.89−2.83 (m, 2H), 2.77 (dd, *J* = 16.4, 7.2 Hz, 1H), 2.65 (dd, *J* = 15.6, 6.5 Hz, 1H), 2.55−2.49 (m, 2H), 1.96−1.87 (m, 1H), 1.76−1.67 (m, 1H), 1.65−1.53 (m, 2H). HRMS (ESI) *m/z* calculated for $C_{31}H_{46}N_9O_{14}$ $(M+H)^+$: 768.3101, found: 768.3164.

STABILITY STUDIES.
T4M(1-[β-D-Glc])-OH (**11**) (50 mg, 0.07 mmol) and N-1-gluco-Asn (15) (50 mg, 0.07 mmol) were dissolved in 3 mL of 2 M HCl (aq), 4 M HCl (aq), 2 M NaOH (aq), 2.6 M HCl/EtOAc or Et_3N, and stirred for the time and temperature indicated in Table 1. The relative stability of the substrates was determined by TLC analysis and integration of ^1H NMR signals of side-products and starting material.

SOLID PHASE $\alpha_v\beta_3$ BINDING ASSAY
The affinity of c(RGDfV), c(RGDyV), c(RGDy-[*N*-1-β-gluco-Asn]), and c(RGDy-T4M(1-[β-D-Glc])) for the $\alpha_v\beta_3$ integrin was determined in a competitive binding assay using the dimeric peptide ^{111}In-DOTA-E-[c(RGDfK)]$_2$ as a tracer. The ^{111}In-labeled peptide (3 MBq/μg) was prepared as described earlier.[20] Microtiter 96-well vinyl assay plates (Corning B.V., Schiphol-Rijk, The Netherlands) were coated with 100 μL/well of a solution of purified human integrin $\alpha_v\beta_3$ (150 ng/mL) (Chemicon International, Temecula, CA, USA) in a coating buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mM $MnCl_2$) for 17 h at 4 °C. The plates were washed twice with binding buffer (0.1% bovine serum albumin (BSA) in coating buffer). The wells were blocked for 2 h with 200 μL blocking buffer (1% BSA in coating buffer) at room temperature. The plates were washed twice with binding buffer. Then 100 μL binding buffer containing 11 kBq of ^{111}In-DOTA-E-[c(RGDfK)]$_2$ and appropriate dilutions of nonlabeled c(RGDfV), c(RGDyV), c(RGDy-[*N*-1-β-gluco-Asn]), and c(RGDy-T4M(1-[β-D-Glc])) in binding buffer were incubated in wells at 37 °C for 1 h. After incubation, the plates were washed three times with binding buffer. The retained radioactivity in each well was determined in a γ-counter. IC_{50} values of the RGD peptides were calculated by non-linear regression using GraphPad Prism (GraphPad Prism 4.0 Software, San Diego, CA, USA). Each data point is the average of three determinations.

RADIOLABELING OF THE RGD PEPTIDES
The peptides containing tyrosine, c(RGDyV), c(RGDy-[*N*-1-β-gluco-Asn]), and c(RGDy-T4M(1-[β-D-Glc])) were labeled with ^{125}I using the Iodogen method. The peptide (10–20 μg) was dissolved in 100 μL phosphate-buffered saline (PBS) (pH 7.4) in a 1.5 mL polypropylene vial coated with 100 μg Iodogen and [^{125}I]-NaI (37 MBq) was added to the vial. After 10 min, the solution was transferred to another vial to terminate the iodination. A sample of the mixture was analyzed by HPLC. Radiochemical purity was always higher than 98%.

BIODISTRIBUTION STUDIES
In the right flank of 6-8 week old female nude BALB/c mice, 0.2 mL of a suspension of 15 x 10^6 cells/mL SK-RC-52 cells was injected subcutaneously (s.c.). Two weeks after inoculation of the tumor cells, mice were randomly divided into four groups. Mice were injected with ^{125}I-c(RGDfV) (0.5 MBq, 0.5 μg) , ^{125}I-c(RGDyV) (0.5 MBq, 0.5 μg), ^{125}I-c(RGDy-[*N*-1-β-gluco-Asn]) (0.5 MBq, 0.5 μg), or ^{125}I- c(RGDy-T4M(1-[β-D-Glc])) (0.5 MBq, 0.5 μg) *via* the tail vein. Mice were euthanized by CO_2 asphyxiation 2 h postinjection (p.i.) (4 mice/group). Blood, tumor and all the major organs and tissues were dissected, weighted, and counted in a well-type γ-counter. The percentage injected dose per gram (%ID/g) was determined for each sample. To investigate whether the uptake of each of the 4 RGD peptides (in the dissected tissues) was $\alpha_v\beta_3$ mediated, a separate group of mice was coinjected with an excess (50 μg) of non-radiolabeled DOTA-E-[c(RGDfK)]$_2$ to saturate all the $\alpha_v\beta_3$ integrin receptors, and the

biodistribution of the radioiodinated RGD peptide-conjugates was determined as described above. All animal experiments were approved by the local animal welfare committee in accordance with Dutch legislation and carried out in accordance with their guidelines.

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SUMMARY

Triazole-linked Glycosyl Amino Acids and Peptides

Naturally occurring glycosylated peptides play an important role in various biological processes and are therefore interesting lead molecules for the preparation of new therapeutic drugs. Synthesis of these natural glycopeptides is frequently hampered by the sensitivity of the natural glycosidic linkage towards acidic and basic conditions. Another important pathway for glycopeptide degradation involves enzymatic cleavage of the carbohydrate moiety.

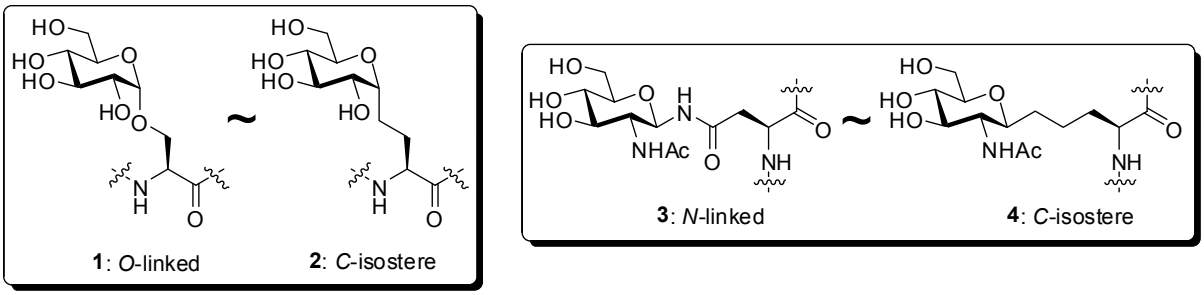
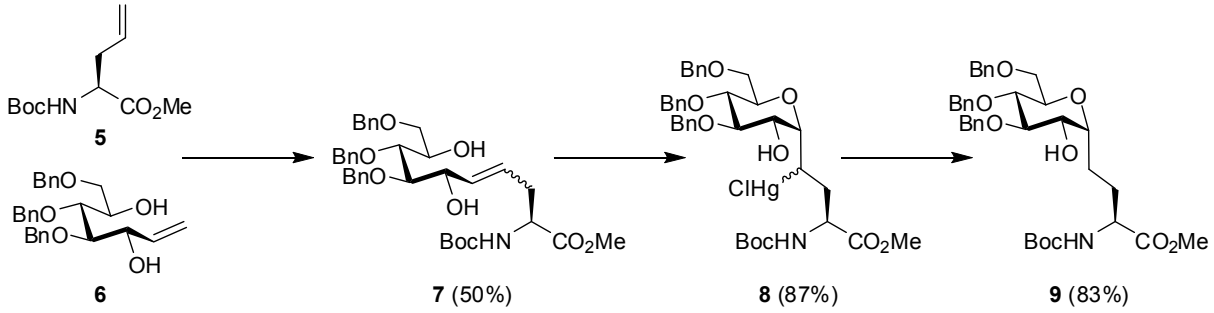


Figure 1. *Natural glycosidic linkage and their C-linked analogues.*

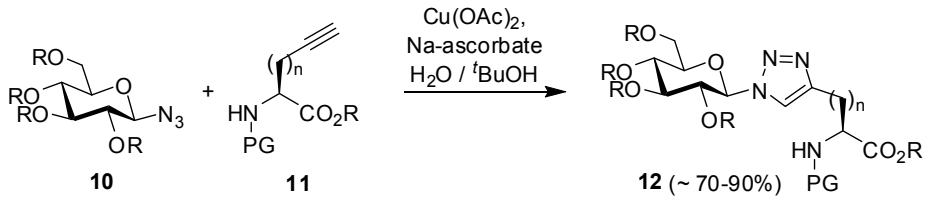
A search for new, more stable mimics led to the development of *C*-linked isosteres, providing excellent chemical and enzymatic stability without negatively influencing the biological properties (Figure 1). A general introduction of natural glycopeptides as well as an overview of recent synthetic approaches for the preparation of new glycosidic amino acid isosteres is given in Chapter 1.

Chapter 2 details our efforts to prepare stable glycosidic *C*-linked serine isosteres by ligation of an open-chain unsaturated sugar to an allylglycine derivative, producing acyclic glycoamino acid alkenes. Subsequent mercury(II)-induced cyclization followed by a reduction led to the desired product, a mimic of the natural *O*-linked glycoamino acids with the proper number of interconnecting atoms. An example hereof is presented in Scheme 1. The resulting *C*-linked glycosyl serine analogues, obtained in a few straightforward steps, are known to surpass the natural *O*-linked analogues in stability.



Scheme 1. *Cross metathesis / mercury(II)-induced cyclization strategy.*

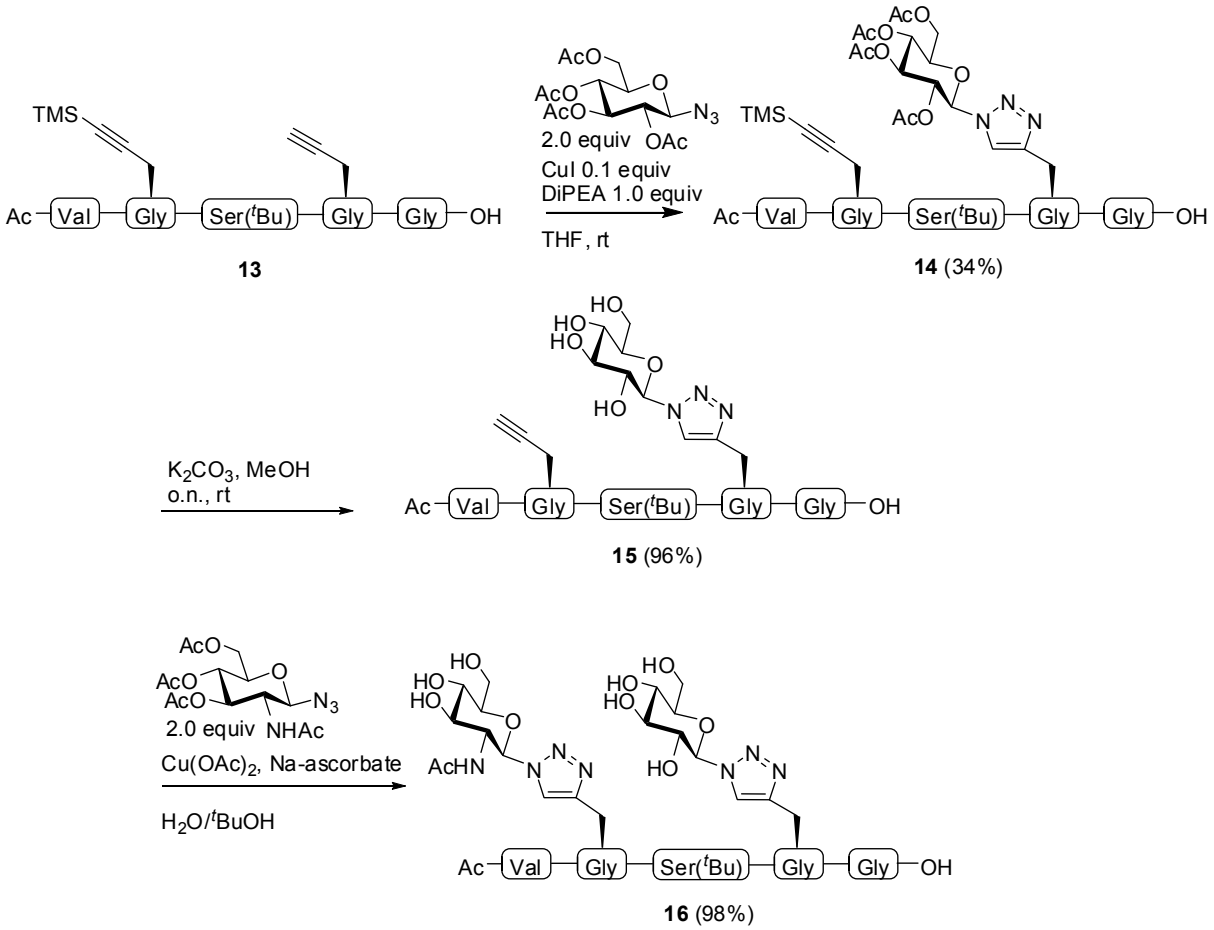
Chapter 3 discloses a novel, expedient, high-yielding synthesis of triazole-linked glycopeptides via Cu(I)–catalyzed [3+2] cycloaddition of azido–functionalized glycosides and acetylenic amino acids (Scheme 2). The resulting triazole-linked products, mimicking the *N*-linked glycosides found in Nature, have potentially relevant biological properties. By application of this method a small library of glycosidic amino acids was prepared. Both α- and β-configured triazolyl glycopeptides were accessible by combination of a variety of azido-functionalized glycosides with a range of acetylenic amino acids, resulting in a novel class of stable glycopeptide mimics.



Scheme 2. *Cu(I)–catalyzed [3+2]-cycloaddition leading to triazolyl glycosidic amino acids.*

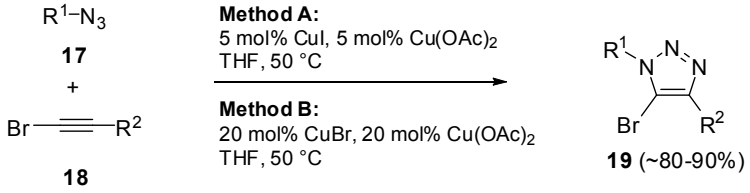
In Chapter 4, a range of protective groups were examined for application in an orthogonal coupling procedure *en route* to triazole analogues of natural glycopeptides with multiple glycosylated side-chains. Both TMS- and TBDPS-protected propargylglycines were prepared via an alkylation strategy, subsequent enzymatic resolution afforded the enantiopure TMS-derivative. Subsequently, a variety of pentapeptides, bearing a free as well as a protected (TMS or TBDPS) acetylene side-chain, were prepared via solid-phase chemistry. A stepwise [3+2]

cycloaddition, deprotection and again [3+2] cycloaddition, either on resin or in solution (Scheme 3) afforded the desired diglycosylated glycopeptides.



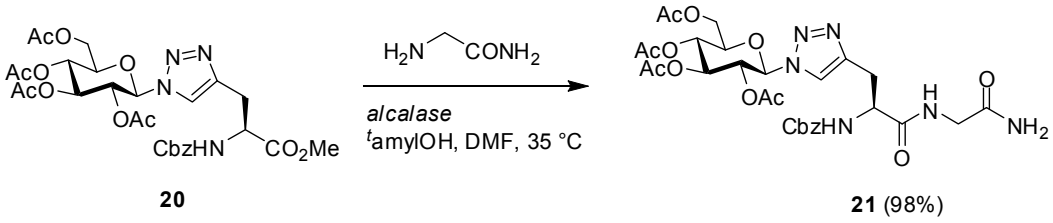
Scheme 3. *Orthogonal coupling procedure en route to triazole analogues of natural glycopeptides.*

In Chapter 5 a novel copper-catalyzed cycloaddition of bromoalkynes and organic azides is described (Scheme 4). Copper-mediated coupling resulted in the formation of bromo-containing trisubstituted [1,2,3]-triazole derivatives in high yield and a regioselective manner, and was applied in the cycloaddition of a variety of bromoacetylenes and azides. The bromide substituent in the resulting triazoles provided a versatile synthetic handle for further functionalization to trisubstituted [1,2,3]-triazoles.



Scheme 4. *Novel copper-catalyzed cycloaddition of bromoalkynes and organic azides.*

The chemoenzymatic peptide coupling of glycosylated triazolyl- and amide-linked amino acid methyl esters, effected by *alcalase*, a proteolytic enzyme mixture produced by *Bacillus licheniformis* is described in Chapter 6. Both the common amide-linked as well as the unnatural triazole-linked substrates were ligated to amino acid amides in moderate to good yields, producing dipeptides. An example is depicted in Scheme 5. Moreover, a first study towards the chemoenzymatic glycosylation of triazole-linked glycopeptides is presented.



Scheme 5. *Enzymatic peptide coupling of glycosylated triazolyl-linked amino acid methyl ester.*

Chapter 7 describes the preparation of several cyclic RGD derivatives by a combination of solid phase and solution phase synthesis for selective targeting of $\alpha_v\beta_3$ integrin expressed in tumors (Figure 2). In order to evaluate the value of a triazole moiety as an amide isostere, the side-chain glycosylated RGD peptides were synthesized with either a natural amide linkage or a triazole. Affinity of the RGD constructs for $\alpha_v\beta_3$ integrin was determined in a solid phase competitive binding assay, showing strong similarity in binding affinity for each of the compounds under evaluation.

Furthermore, the *in vivo* tumor targeting potential of glycosylated RGD peptides, linked via amide or triazole, was investigated by determining the biodistribution of ^{125}I-labeled derivatives in mice with tumors expressing $\alpha_v\beta_3$. All of the RGD derivatives showed preferential uptake in the subcutaneous tumors, with the highest tumor-to-blood ratio measured for the triazole-linked glycosylated derivative. The

results of the present study are a clear indication of the value of the triazole moiety as a suitable amide isostere in the development of glycosylated peptides as pharmaceuticals.

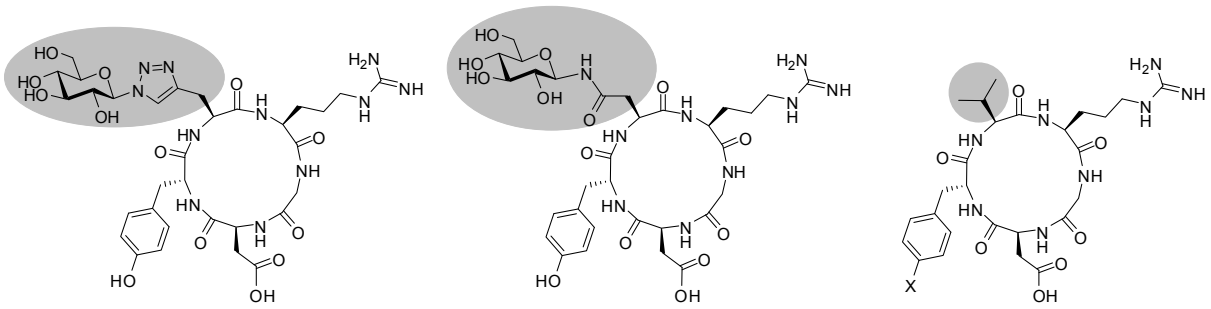
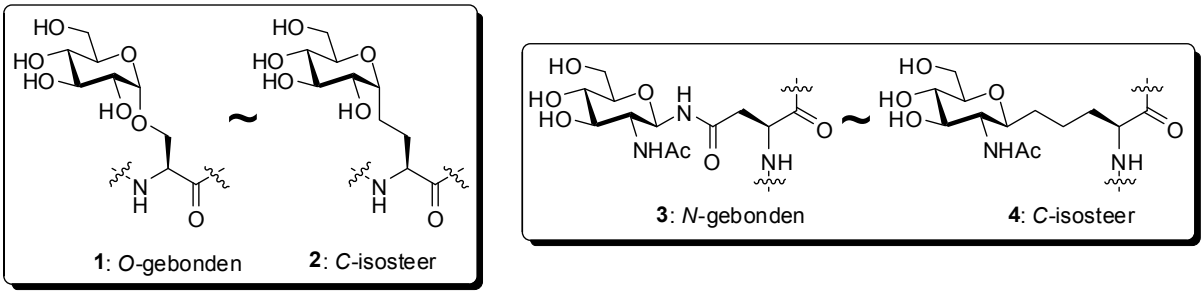


Figure 2. *Cyclic RGD peptides for selective integrin targeting.*

SAMENVATTING

Triazool gebonden glycosylaminozuren en -peptiden

Glycopeptiden zijn eiwitten die post-translationeel gefunctionaliseerd zijn met een of meerdere (complexe) koolhydraten. Natuurlijk voorkomende glycopeptiden spelen een belangrijke rol in diverse biologische processen en vormen bijgevolg een mogelijk interessant uitgangspunt voor de ontwikkeling van nieuwe geneesmiddelen. De synthese van natuurlijk voorkomende glycopeptiden wordt vaak bemoeilijkt door de gevoeligheid van de glycosyl-peptide acetaalbinding voor zure en basische omstandigheden. Verder kunnen ook enzymatische processen leiden tot hydrolyse van de acetaalbinding en zo aanleiding tot afsplitsing van het koolhydraatdeel.

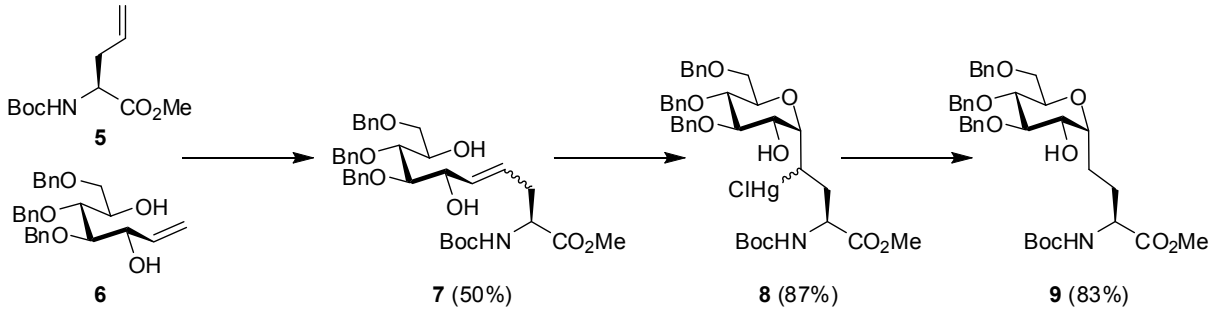


Figuur 1. *Natuurlijk voorkomende acetaalbindingen in glycopeptiden en bijbehorende C-analoga.*

Onderzoek naar nieuwe stabiele mimetica leidde onder meer tot de ontwikkeling van *C*-isosteren met uitstekende chemische en enzymatische stabiliteit, zonder de biologische eigenschappen negatief te beïnvloeden (Figuur 1). In hoofdstuk 1 wordt een algemene inleiding over natuurlijk voorkomende glycopeptiden gegeven, evenals een overzicht van recent gepubliceerde routes voor de synthese van glycosylaminozuur isosteren.

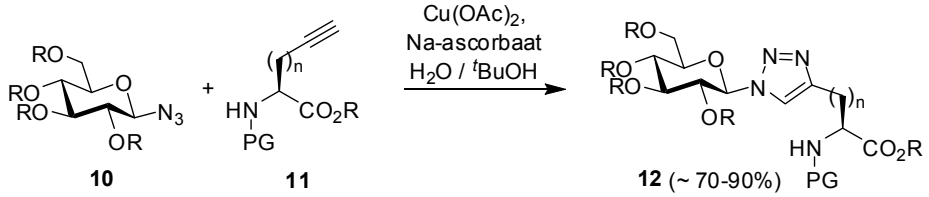
Hoofdstuk 2 beschrijft de inspanningen om stabiele *C*-gebonden glycosylserine-isosteren te synthetiseren via cross-metathese van onverzadigde lineaire suikers aan allylglycine derivaten. De resulterende lineaire conjugaten kunnen vervolgens via een kwik(II)-geïnduceerde ringsluiting, gevolgd door een reductie tot de gewenste producten leiden. De zo verkregen mimetica van de natuurlijke *O*-gebonden

glycoaminozuren bevatten nu het juiste aantal bindingsatomen, waarvan een voorbeeld in Schema 1 is weergegeven. Uit de literatuur is reeds bekend dat dergelijke *C*-gebonden glycosylserine analoga stabieler zijn dan de natuurlijke *O*-gebonden derivaten.



Schema 1. *Cross-methathese/kwik-geïnduceerde ringsluitingsstrategie.*

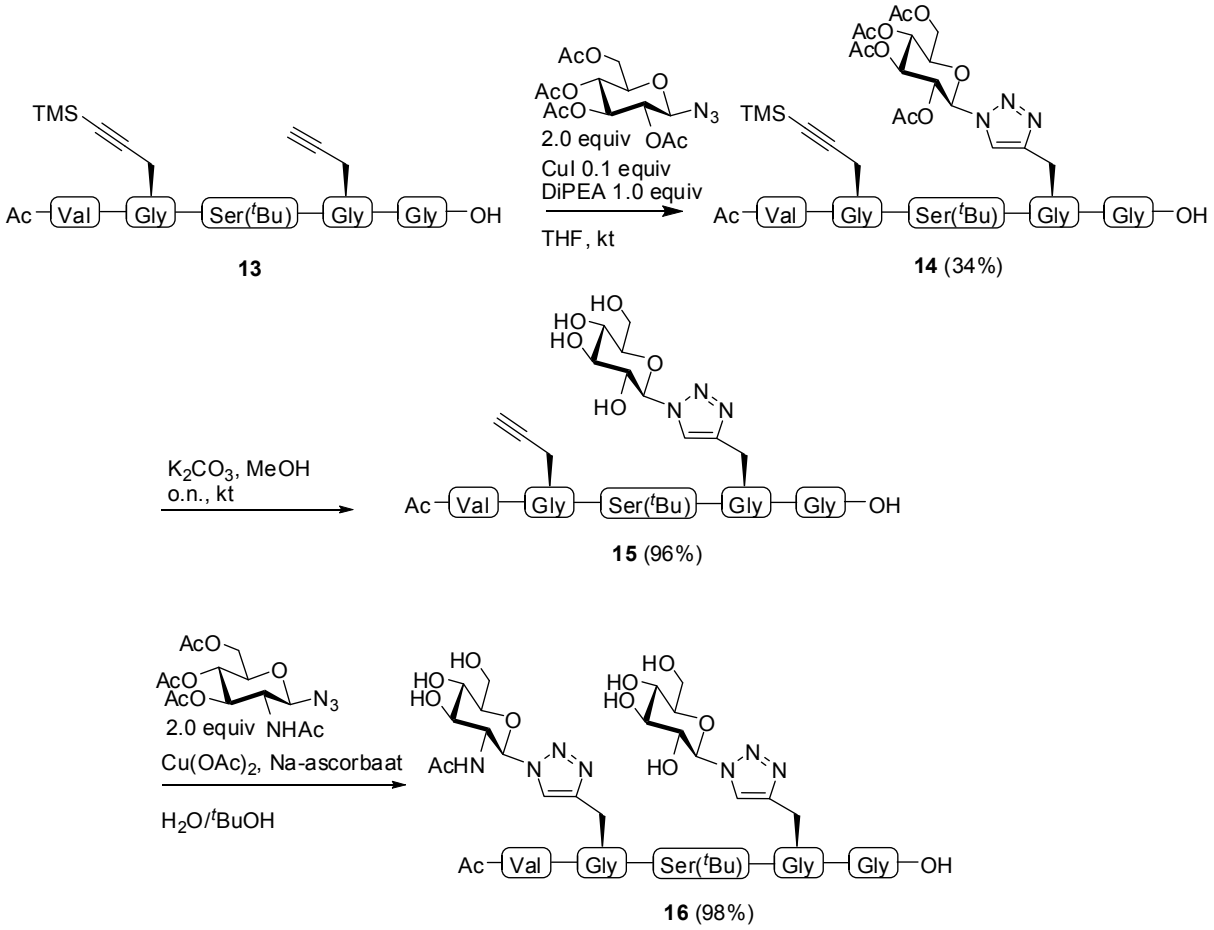
Hoofdstuk 3 behandelt een effectieve synthese van nieuwe triazool-gebonden glycopeptiden via een Cu(I)-gekatalyseerde 1,3-dipolaire cycloadditie van azide-bevattende glycosiden en aminozuur-houdendeacetylenen (Schema 2). De resulterende triazool-gebonden producten, mimetica van de natuurlijke *N*-gebonden glycosiden, hebben mogelijk relevante biologische eigenschappen. Bijgevolg werd via deze methode een kleine bibliotheek van glycosylaminozuur analoga gesynthetiseerd. Zowel α– als β-triazolylglycopeptiden waren toegankelijk door een variëteit van azide-gefunctionaliseerde glycosiden met aminozuuracetylenen te combineren, wat tot deze geheel nieuwe klasse van stabiele glycopeptiden leidde.



Schema 2. *Synthese van triazolyl-glycopeptiden via Cu(I)-gekatalyseerde 1,3-dipolaire cycloadditie.*

In hoofdstuk 4 is een reeks beschermgroepen onderzocht op hun toepasbaarheid in orthogonale koppelingsprocedures, met als doel triazool-bevattende analoga van natuurlijke glycopeptiden met meervoudig geglycosyleerde zijketens eenvoudig toegankelijk te maken. Zowel TMS- als TBDPS-beschermde propargylglycinederivaten werden via een alkylering gesynthetiseerd en vervolgens

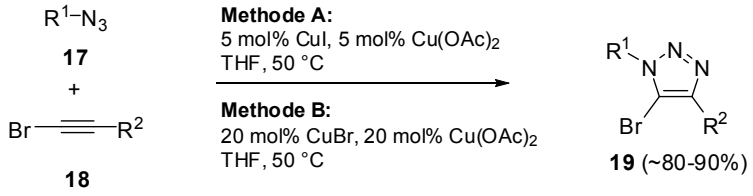
kon via enzymatische resolutie het TMS derivaat omgezet worden in het overeenkomnstige enantiozuivere aminozuur. Aansluitend werden verschillende pentapeptiden, met een vrije én een beschermde acetyleen zijgroep, bereid via synthese op een vast dragermateriaal. Stapsgewijze 1,3-dipolaire cycloadditie, ontscherming gevolgd door nogmaals 1,3-dipolaire cycloadditie, op de vaste fase of in de vloeistoffase (Schema 3) gaf de gewenste tweevoudig geglycosyleerde glycopeptiden.



Schema 3. *Orthogonale koppelingsprocedure voor de synthese van triazool analoga van natuurlijke glycopeptiden.*

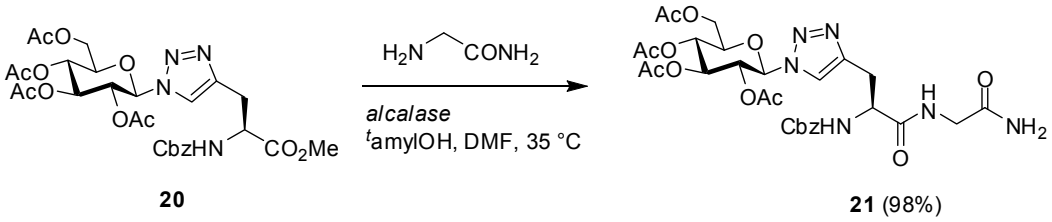
In Hoofdstuk 5 wordt een nieuwe koper-gekatalyseerde cycloadditie van niet eindstandige broomalkynen en organische aziden beschreven (Schema 4). Deze koper-geinduceerde koppeling, resulterend in de vorming van broom-bevattende trigesubstitueerde [1,2,3]-triazoolderivaten (in hoge opbrengst en goede regioselectiviteit), werd toegepast voor de cycloadditie van een reeks broom-acetylenen en aziden. De broom-substituent in de verkregen triazolen kan benut

worden als een synthetisch handvat voor verdere functionalisering tot andere trigesubstitueerde [1,2,3]-triazolen.



Schema 4. *Nieuwe koper-gekatalyseerde cycloadditie van broomalkynen en organische aziden.*

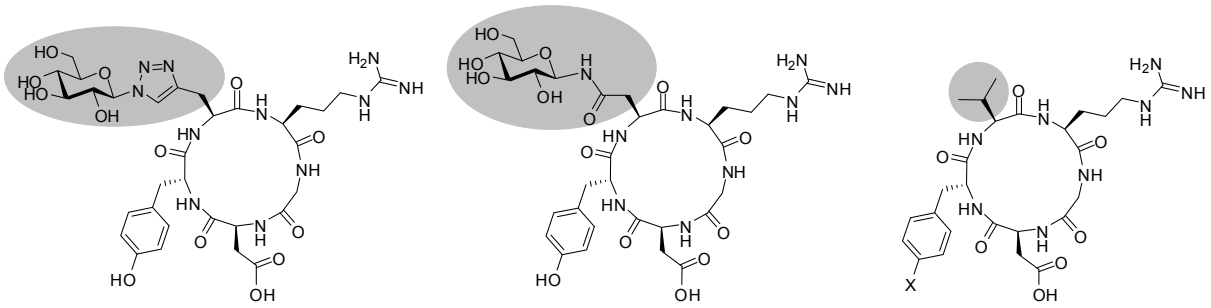
De chemoenzymatische peptidekoppeling van geglycosyleerde triazool- en amide-gebonden aminozure methylesters, geinduceerd door *alcalase* (een proteolytisch enzymmengsel geproduceerd door *Bacillus licheniformis*), wordt beschreven in Hoofdstuk 6. Zowel de amide-gebonden als de niet-natuurlijke triazool-gebonden substraten werden aan aminozuuramiden gekoppeld, wat leidde tot dipeptiden in een matige tot goede opbrengst. Een voorbeeld is weergegeven in Schema 5. Bovendien wordt een eerste studie naar chemoenzymatische glycosylering van triazool-gebonden glycopeptiden behandeld.



Schema 5. *Enzymatische peptidekoppeling van geglycosyleerde triazool-gebonden aminozure methylesters.*

Hoofdstuk 7 beschrijft de synthese van diverse cyclische RGD derivaten, middels een combinatie van vaste en vloeistoffase chemie. Deze peptiden zijn zeer geschikt voor het selectief targeten van in tumoren tot expressie gebrachte $\alpha_v\beta_3$ integrine receptoren (Figuur 2). Om de biologische waarde van een triazool als amide isosteer te evalueren, werden zijketen-geglycosyleerde RGD peptiden met respectievelijk een amide en triazool binding gesynthetiseerd. De affiniteit van de verkregen RGD derivaten voor de $\alpha_v\beta_3$ integrine receptor werd *in vitro* bepaald in een ELISA assay, welke aantoonde dat de bindingsaffiniteit van de geëvalueerde verbindingen nagenoeg gelijk was.

Het *in vivo* tumor targeting potentieel van de geglycosyleerde RGD peptiden (met een amide of triazool binding) werd onderzocht door de bioverdeling van ^{125}I-gelabelde RGD derivaten in muizen met $\alpha_v\beta_3$ bevattende tumoren te bepalen. De RGD derivaten werden in muizen met onderhuidse tumoren ingebracht, waarna vervogens de muizen werden gedood en de radioactiviteit in de verschillende organen en de tumor werd gemeten. Het triazool-bevattende glycoRGD derivaat bleek, evenals het overeenkomstige amide derivaat, voor het grootste deel in de tumor terecht te komen. Verder werd voor het triazool-gebonden geglycosyleerde derivaat de hoogste tumor-tot-bloed verhouding werd gemeten. De resultaten van dit onderzoek vormen daarmee een duidelijke aanwijzing voor de potentie van de triazool als geschikt amide isosteer in biologische systemen.



Figuur 2. *Cyclische RGD peptiden voor het selectief targeten van integrine receptoren.*

DANKWOORD

Wow, als je hier bent aangekomen heb je al aardig wat uurtjes in mijn proefschrift gelezen, respect! Of is dit soms het eerste wat je leest? Het dankwoord, wellicht een van de lastigste gedeeltes van het schrijven van een proefschrift, als je niet iemand vergeet, spel je wel iets verkeerd. Toch is het een belangrijk deel van mijn proefschrift, want hier krijg ik de kans om mijn dank uit te spreken aan een ieder die in fysieke, dan wel morele steun of afleiding heeft bijgedragen aan mijn promotie.

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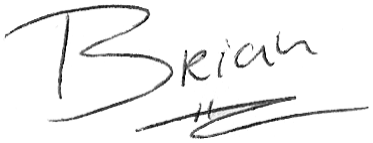

Brian Kuijpers

Nijmegen, 25-5-2008

CURRICULUM VITAE

Brian Kuijpers werd geboren op 12 november 1975 te Heerlen. Na het behalen van het HAVO diploma aan het Eijkhagen College te Landgraaf, startte hij in 1994 met het Hoger Laboratorium Onderwijs (HLO) aan de Hogeschool Zuid te Sittard. In 1999 studeerde Brian, na een afstudeerstage bij Dex-Plastomers te Geleen, af in de richting van Organische Chemie. Alvorens aan te vangen met de studie scheikunde aan de toenmalige Katholieke Universiteit Nijmegen, was Brian voor een korte periode werkzaam bij DSM Petrochemicals te Geleen. Tijdens de studie scheikunde werd een hoofdvakstage getiteld "Synthesis of Enantio- and Diastereoselective Amino Acids with multiple Stereocenters via the *aza*-Claisen rearrangement" uitgevoerd in de vakgroep Synthetisch Organische Chemie onder leiding van Prof. F.P.J.T. Rutjes. Begin 2003 werd het doctoraal examen behaald en vanaf maart 2003 tot maart 2007 was hij aangesteld als Junior Onderzoeker in dezelfde vakgroep. Tijdens deze periode werd het in dit proefschrift beschreven onderzoek uitgevoerd onder begeleiding van Prof. F.P.J.T. Rutjes en Dr. F.L. van Delft. Momenteel is Brian Kuijpers werkzaam als Senior Scientist bij Mercachem B.V. te Nijmegen.

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Journal articles:

- B.H.M. Kuijpers, S. Groothuys, C. Hawner, J. ten Dam, P.J.L.M. Quaedflieg, H.E. Schoemaker, F.L. van Delft, F.P.J.T. Rutjes, Cu-Catalyzed Formation of Triazole-Linked Glycoamino Acids and Application in Chemoenzymatic Peptide Synthesis, *Org. Process Res. Dev.*, **2008**, 12, 503-511.

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Patent:

- B.H.M. Kuijpers, S. Groothuys, F.L. Van Delft, F.P.J.T. Rutjes, R.H. Blaauw, Triazole linked glycoamino acids and glycopeptides. *WO2005118625*, **2005**.

Presentations:

- "Metal-mediated synthesis of stable glycopeptides mimics"; *oral presentation*, 232nd American Chemical Society National Meeting, San Fransisco, USA (2006)

- "Metal-mediated synthesis of stable glycopeptides mimics"; *oral presentation*, Netherlands Catalysis and Chemistry Conference VI (NCCCVI), Noordwijkerhout, The Netherlands (2005)

- "Synthesis of triazole-linked glycopeptide mimics"; *oral presentation*, CW/NWO study group meeting Design and Synthesis, Lunteren, The Netherlands (2004)